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Enrichment of Triadic and Terminal Cisternae Vesicles from Rabbit Skeletal Muscle

J.W. Kramer, D.G. Ferguson[1], A.M. Corbett
Department of Physiology and Biophysics, Wright State University, Dayton, OH 45435 and
[1]Department of Anatomy, Case Western Reserve University, Cleveland, OH 44106, USA

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Abstract. An enriched triad and terminal cisternae preparation was achieved from skeletal muscle through alterations of the differential centrifugation and muscle homogenization protocols. Both yield and specific activity (pmoles of radioligand binding per mg protein) were optimized for ^{3}H-PN200-l10 (transverse tubule marker) and ^{3}H-ryanodine (terminal cisternae marker) binding sites. By pelleting crude microsomes between 2,000 an 12,000 × *g* without any rehomogenizations, we improved both the yield and specific activity of transverse tubule and terminal cisternae markers in crude microsomes by approximately 4-fold to 1000–3000 pmoles binding sites (starting material: approximately 400 grams wet weight fast twitch skeletal muscle), with 10–15 pmoles/mg. Rehomogenization of the 1,000 × *g* pellet, which is typically discarded, allowed recovery of an additional 5000 pmoles PN200-110 binding sites and an additional 8000 pmoles ryanodine binding sites. Crude microsomes from the rehomogenized 1,000 × *g* pellets typically displayed specific activities of 20–25 pmoles binding/mg for both ^{3}H-PN200-110 and ^{3}H-ryanodine. Separation of crude microsomes on a sucrose gradient increased specific activity up to a maximum of 50 pmoles/mg in a specific fraction, a five- to ten-fold increase over standard triadic or terminal cisternae preparations. The mean specific activity for enriched triads was 30–40 pmoles/mg for both PN200-110 and ryanodine in pooled fractions, while pooled fractions of enriched terminal cisternae displayed low ^{3}H-PN200-110 binding (3–5 pmoles/mg) and high ^{3}H-ryanodine-specific activity (30–40 pmoles/mg).

Key words: Triad — Terminal cisternae — Sarcoplasmic reticulum — Skeletal muscle — Dihydropyridine receptor — Ryanodine receptor

Correspondence to: A.M. Corbett; email: adrian.corbett@wright.edu

Introduction

Excitation-contraction coupling in skeletal muscle is known to involve two Ca^{2+} channels, the dihydropyridine receptor of the transverse tubules (Ríos & Brum, 1987) and the ryanodine receptor of the sarcoplasmic reticulum (Kawamoto et al., 1986; Inui et al., 1987; Campbell et al., 1987; Lai et al., 1987, 1988). Upon depolarization, the dihydropyridine receptor is thought to undergo a conformational change that is transmitted to the ryanodine receptor by an unknown mechanism (Ríos, Ma & Gonzalez, 1991), and thereby induces the release of calcium from the sarcoplasmic reticulum.

The triad, composed of one transverse tubule and two physically joined sarcoplasmic reticulum cisternae, is the elementary unit of excitation-contraction coupling. It contains all the components essential for this process. It has been shown that vesicles composed of membrane from the triad region of the muscle can be isolated using different homogenization and centrifugation techniques (Caswell, Lau & Brunschwig, 1976; Campbell, Franzini-Armstrong & Shamoo, 1980; Mitchell, Palade & Fleischer, 1983; Ikemoto, Antoniu & Kim, 1984; Meissner, 1984; Saito et al., 1984; Kramer & Corbett, 1995). The triad vesicle preparation contains all of the proteins necessary to study both calcium-induced calcium release and depolarization-induced calcium release in skeletal muscle. It also offers the unique advantage that full control over the media in contact with the cytoplasmic face of channels and the full triad junction can be maintained. Therefore, it is a simple matter to study the effects of different modifiers on the process of excitation-contraction coupling. Other investigators have relied on vesicle preparations in order to study the effects of different modulators on calcium-induced calcium release from isolated terminal cisternae vesicles (Ohnishi, 1979; Yamamoto & Kasai,

1982; Morii and Tonomura, 1983; Nagasaki & Kasai, 1983; Kim, Ohnishi & Ikemoto, 1983; Meissner, 1984; Ikemoto, Antoniu & Mészáros, 1985; Meissner, 1986; Meissner, Darling & Eveleth, 1986). Still others have used isolated vesicles from skeletal muscle as starting material for purification of the skeletal muscle dihydropyridine receptor (Curtis & Catterall, 1984; Borsotto et al., 1985; Flockerzi et al., 1986; Morton & Froehner, 1987; Takahashi et al., 1987; Leung et al., 1987), voltage-gated sodium channels (Barchi, 1983; Kraner, Tanaka & Barchi, 1985; Casadei, Gordon & Barchi, 1986) and ryanodine receptor (Kawamoto et al., 1986; Inui, Saito & Fleischer, 1987; Lai et al., 1987, 1988; Campbell et al., 1987; Hawkes, Díaz-Muñoz & Hamilton, 1989).

There are two important steps in a skeletal muscle vesicle preparation protocol: 1) the disruption of the muscle membrane and 2) the separation of the membrane vesicles. Typically, disruption is performed using a Waring blender, Polytron tissumizer and/or a meat grinder. Membrane vesicle formation occurs spontaneously upon disruption. Separation of the membrane vesicles is performed by variable centrifugation, which separates vesicles that have large differences in their sedimentation rates. Vesicles with a greater rate of sedimentation can be pelleted, while other vesicles remain in the supernatant. In skeletal muscle vesicle preparations, a lower-speed centrifugation results in the pelleting of contractile proteins, sheets of sarcolemma, nuclei and mitochondria. The supernatant from this low-speed cut can then be centrifuged at much higher speeds to pellet crude microsomes, which contain sarcoplasmic reticulum (SR), sarcolemma, transverse tubules (t-tubules) and triadic vesicles.

Many investigators have developed procedures to isolate skeletal muscle vesicles ranging from transverse tubules to triadic vesicles. Of the many different procedures, all use a low-speed centrifugation to separate contaminating membranes from the freshly homogenized muscle. Many investigators have performed the centrifugation using speeds from 10,000 – 14,300 $\times$ g (Caswell et al., 1976; Mitchell et al., 1983; Hawkes, Díaz-Muñoz & Hamilton, 1989; Kim et al., 1990; Ohkusa et al., 1991; Bers & Stiffel, 1993). However, other investigators have performed this initial centrifugation at a much lower speed of about 3000 $\times$ g (Campbell et al., 1980; Fernandez, Rosemblatt & Hildalgo, 1980; Meissner, 1984). The supernatant that is recovered is then centrifuged at higher speeds to pellet the crude microsomes. The speed used for this centrifugation typically ranges from 95,000–130,000 $\times$ g (Caswell et al., 1976; Fernandez et al., 1989; Hawkes et al., 1989; Kim et al., 1990). However, in other studies, speeds under 50,000 $\times$ g have been used for this second centrifugation (Campbell et al., 1980; Kim, Ohnishi & Ikemoto, 1983; Meissner, 1984; Ohkusa et al., 1991; Bers & Stiffel, 1993). After the crude microsomes have been isolated, they can be further purified through separation on continuous or discontinuous sucrose gradients (Caswell et al., 1976; Campbell et al., 1980; Mitchell et al., 1983; Hawkes et al., 1989; Corbett et al., 1992; Bers & Stiffel, 1993). Mitchell et al. (1983) used enzymatic assays to estimate the purity of the triad vesicle preparation and compared their results to those obtained by Caswell et al. (1976) and Rosemblatt et al. (1981). They determined that they were able to isolate a population of triadic vesicles that was significantly purer; however, their protocols required the addition of pyrophosphate to achieve the greater purity. Treatment with pyrophosphate has subsequently been determined to eliminate the ability of these vesicles to release calcium upon depolarization (Corbett et al., 1992). Other protocols have used the binding of ryanodine (Corbett et al., 1992; Bers & Stiffel, 1993; Anderson, Cohn & Meissner, 1994) and nitrendipine or PN200-110 (Corbett et al., 1992; Bers & Stiffel, 1993; Anderson et al., 1994; Kramer & Corbett, 1995) to gauge the purity of the isolated membrane vesicles. However, the specific activity for these radioligand binding assays was rather low (5–10 pmoles binding per mg protein) for the triadic fractions that were isolated.

Therefore, in this study we have attempted to isolate a partially purified population of triadic vesicles that displayed a high specific activity of both PN200-110 and ryanodine radioligand binding. In our first set of experiments, we examined the effect of different centrifugation cuts on the yield of terminal cisternae and triads. We varied the centrifugal force used to isolate crude microsomes from the homogenized skeletal muscle and discovered that lowering the speed of the initial low-speed centrifugation as well as the pelleting force for the crude microsomes liberated many more triadic vesicles with higher specific activity. We next investigated whether homogenization of the muscle could be improved to yield more enriched triads and terminal cisternae. We determined that the traditional method of a single disruption using the Waring blender was not efficient at liberating all of the potential triads from the skeletal muscle. In fact, we found that a significant amount of additional triads could be liberated by exposing the muscle to additional homogenizations using a Polytron tissumizer. In addition, the morphology of our isolated vesicles was studied by electron microscopy.

Materials and Methods

The triad vesicles were prepared from the fast-twitch leg muscle of 5–7 lb New Zealand white rabbits. The leg fast-twitch muscles

were removed and minced into 500 ml of ice cold Sucrose-EDTA buffer (250 mM sucrose, 2 mM EDTA, pH 7.0) containing protease inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 47.9 μg/ml pefabloc SC). All solutions were kept on ice and all centrifugations were performed at 5°C.

Triad Vesicle Preparation

The following protocol represents the optimized version for best yield of both enriched triads and terminal cisternae. Alternate versions tested are described in figure legends.

The minced muscle was homogenized in a Waring Blender (2 times at 30 seconds on and 30 seconds off) and, the homogenate was subjected to a $1{,}000 \times g$ spin in a Beckman JA-10 rotor for 20 min. The pellet was retained for a second homogenization and the supernatant poured through three layers of cheesecloth to remove floating material. The pH of the supernatant was adjusted to 7.0 with NaOH. The supernatant was centrifuged at $2{,}000 \times g$ for 30 minutes, and the resulting pellet discarded. The supernatant was centrifuged at $12{,}000 \times g$, and the resulting pellet was washed and rehomogenized in sucrose- histidine buffer (250 mM sucrose, 20 mM histidine, pH 7.0). The supernatant from this spin was centrifuged at $125{,}000 \times g$. The pellet from this final spin was resuspended in sucrose histidine as described above and the supernatant was discarded.

The pellet from the initial $1{,}000 \times g$ centrifugation was resuspended in sucrose EDTA buffer (same volume as original homogenization) and rehomogenized in a protocol using a Polytron Tissumizer (setting = 4000 rpm, 3 × 10 s on, 1 min rest). The chamber holding the homogenate was surrounded with ice to prevent the muscle material from heating up. Following an initial $1{,}000 \times g$ centrifugation, the supernatant from this was then subjected to the same sequential centrifugations as above (2,000, 12,000 and $125{,}000 \times g$) and the isolated crude microsomes were rehomogenized and washed at a final $125{,}000 \times g$ spin. For the third homogenization, this process was repeated, rehomogenizing the $1{,}000 \times g$ pellet from the second rehomogenization and following the subsequent steps outlined in this paragraph. The crude microsomes obtained from the 2K, 12K and $125\text{K} \times g$ spins for the first, second and third homogenizations were assayed for protein, ^{3}H-PN200-110 and ^{3}H-ryanodine binding activity.

For optimized triad and terminal cisternae preparation, crude microsomes from the $12\text{K} \times g$ spin were layered onto continuous sucrose gradients (15 – 65% w/w) and spun at $135{,}000 \times g$ in a Beckman SW-28 rotor for 12–17 hr at 5°C. The gradients were fractionated into 2-ml aliquots and fractions of interest were pooled. The pooled fractions were diluted slowly into sucrose-histidine buffer, concentrated through centrifugation ($125{,}000 \times g$ for 45 min at 5°C) and rehomogenized in a small volume of sucrose-histidine buffer. The concentrated samples were divided into small aliquots (250 μl/vial) and quick-frozen by immersing the cryovials in a mixture of acetone and dry ice; samples were stored at −70°C. The pooled fractions were analyzed for protein, ^{3}H-PN200-110, and ^{3}H-ryanodine binding.

Protein Assay

Protein was determined by the method of Bradford (1976), using bovine serum albumin as the standard. The protein determined for a given sample represents the mean of three different dilutions of the sample, each containing two replicates.

Radioligand Binding Assays

Assays for ^{3}H-PN200-110 (t-tubule marker) and ^{3}H-ryanodine binding (terminal cisternae marker) were performed for every microsomal preparation. Approximately 20–100 μg of a given sample was incubated in a 50 mM Tris HCl solution (pH 7.0) that contained 3–10 nM ^{3}H-PN200-110 at room temperature in the dark for 30 min. Nonspecific binding was determined through the addition of 20–40 μM cold nitrendipine. The samples were filtered through glass fiber filters and washed three times with 4.5 mls of 140 mM choline chloride. The filters were counted by liquid scintillation. B_{max} values for specific binding were obtained with the following equation:

$$B_{max} = \text{specific binding} \cdot ((K_D/[\text{free ligand}]) + 1) \quad (1)$$

where the K_D for PN200-110 was 0.4 nM.

^{3}H-ryanodine binding was performed according to the following protocol. An aliquot of the samples (20–100 μg) was incubated in 1 M KCl, 6 mM Na-ATP, 75 μM $CaCl_2$, 10 mM MOPS, pH 7.4, using between 5–10 nM ^{3}H-ryanodine at room temperature for 1 hour. Nonspecific binding was determined by the addition of 10 μM cold ryanodine. Samples were filtered through glass fiber filters and washed three times with 4.5 ml of a wash solution (150 mM KCl, 10 mM HEPES, pH 7.4). Filters were counted by liquid scintillation. B_{max} values were obtained with the above formula, using a K_D for ryanodine of 14 nM.

Electron Micrographs and Rotary Shadowing of Freeze-dried Samples

For thin-section electron microscopy, membrane fractions were pelleted using a microfuge. The supernatant was aspirated and fixative solution consisting of 2.5% glutaraldehyde and 0.1% tannic acid in 0.1M Na cacodylate was added to each tube. After 30 minutes, the pellets were gently lifted from the bottom of the centrifuge tubes and left in fixative for 90 additional minutes. The pellets were then washed in 0.1% Na-cacodylate, postfixed for 60 minutes in 1% OsO_4, washed, dehydrated in a graded series of ethanol and embedded in Epon epoxy resin. Thin sections were cut using a Reichert Ultracut S, stained with lead citrate and uranyl acetate and micrographs were obtained using a Zeiss EM10 or a JEOL 100C.

For rotary shadowing, membrane fractions were prepared using a modification of a previously described technique (Ferguson, Schwartz & Franzini-Armstrong, 1984). Briefly, membranes were diluted to a final concentration of 1 mg/ml and adsorbed to freshly cleaved mica plates. Excess membrane suspension was washed off with 10 mM histidine and the mica was floated face down on a droplet of 1% uranyl acetate. Excess uranyl acetate was washed off the samples using 30% methanol and the samples were placed in the vacuum chamber of a Cressington CFE-100 freeze-etch apparatus. The samples were dried under vacuum for 15 min and rotary-shadowed with platinum at an angle of 20 degrees. Micrographs were obtained from the replicas using a Zeiss EM10.

SDS Polyacrylamide Gels

Gels were run according to the method of Laemmli (1970). Pooled fractions from sucrose gradients were run on 10% SDS-PAGE with a 3% stacking gel, loading 20 μg/well, in the presence of 5% β-mercaptoethanol. A ratio of 75:1 acrylamide:bis was used for both running and stacking gels to facilitate entry of the large molecular weight ryanodine receptor. The gel was run at 125 V constant voltage for approximately 4 hours. Coomassie staining of the gel followed the protocol outlined in the Hoefer Scientific Instruments manual for gel electrophoresis.

Table 1. Radioligand binding for variable-centrifugation crude microsomes

	Centrifugal Force (× *g*)	Total Protein (mg)	PN200-110 (pmol/mg)	Ryanodine (pmol/mg)
A	14K	21.7	10.94 ± 0.23 (237 pmol)	6.37 ± 0.20 (138 pmol)
	17K	9.34	3.25 ± 0.02 (30 pmol)	2.11 ± 0.18 (20 pmol)
	125K	20.56	1.40 ± 0.10 (29 pmol)	0.94 ± 0.03 (19 pmol)
B	5K	214	14.59 ± 0.89 (3122 pmol)	—
	10K	154	15.50 ± 1.27 (2387 pmol)	—
	15K	116	6.25 ± 0.56 (725 pmol)	—
	125K	295	2.01 ± 0.20 (593 pmol)	—

In Table 1*A*, minced muscle was homogenized and two low-speed centrifugations were performed (1K and 10K × *g*). Pellets from these spins were discarded and the supernatant was subjected to three sequential centrifugations (14K, 17K and 125K × *g*). In Table 1*B*, only one low-speed centrifugation was performed, in which the pellet was discarded (1K × *g*). The supernatant from this spin was spun at four sequential centrifugal forces (5K, 10K, 15K and 125K × *g*). The pellets collected from each spin in *A* and *B* were resuspended in sucrose-histidine buffer (*see* Methods) and assayed for protein (Bradford assay), and radioligand binding, as described in Materials and Methods.

Results

Differential Centrifugation

Minced fast twitch skeletal muscle fibers were homogenized in a sucrose EDTA buffer containing protease inhibitors using a Waring blender (3 × 30 s on, 30 s off). The first protocol tested, which discards a pellet obtained at 10,000 × *g*, and then obtains microsomal pellets at 14,000, 17,000 and 125,000 × *g*, is similar to many of the current protocols for triad and terminal cisternae preparation.

The isolated crude microsomes from the above protocol were assayed for protein, ^{3}H-ryanodine-, and ^{3}H-PN200-110-binding to determine the relative amounts of transverse tubules and terminal cisternae present. The specific activities for both ^{3}H-PN200-110 and ^{3}H-ryanodine binding in these isolated microsomes are recorded in Table 1*A*. According to the radioligand-binding assays, the majority of the PN200-110 and ryanodine binding (consistent with triadic vesicles) were isolated in the 14K × *g* centrifugation. These crude microsomes contained the highest specific activity for ^{3}H-PN200-110 (10.94 pmol/mg) and ^{3}H-ryanodine (6.37 pmol/mg).

Meissner (1984) and Pessah et al. (1986) used low-speed spins of 2000–2600 × *g* to clear the microsomes of contaminating contractile proteins. We next investigated the effect of lowering the speeds used to obtain microsomes. Following an initial 1,000 × *g* centrifugation, in which the pellet was discarded, the supernatant was subjected to sequential spins of 5K, 10K, 15K and 125K × *g*, with crude microsomes collected from each spin and assayed for protein and ^{3}H-PN200-110 binding (Table 1*B*). The amount of protein collected from each of the four centrifugations is similar, ranging from 116–295 mg. However, major differences in the number of radioligand binding sites collected at each step are seen. In the 5K and 10K × *g* spins, 3.1 and 2.4 nmoles of ^{3}H-PN200-110 binding sites were recovered respectively: this represents a more than sevenfold increase in the number of sites obtained when a 10K × *g* spin was used prior to collecting microsomes (*see* Table 1*A*). The 15K and 125K × *g* centrifugations pelleted 725 and 593 pmoles of ^{3}H-PN200-110 binding sites, respectively, values which were higher than those obtained for similar centrifugations in Table 1*A*. Overall, 81% of the total number of radioligand binding sites liberated (both PN200-110 and ryanodine) were recovered in spins of 10K × *g* or under. This means that protocols that use a 10K × *g* spin prior to collecting skeletal muscle microsomes, might actually be discarding the majority of their triadic vesicles. We later added an additional centrifugation step of 2,000 × *g*, which was effective at removing actin and myosin contractile proteins without much loss of PN200-110 and ryanodine binding sites (*see* Fig. 3).

Effect of Multiple Homogenizations

The method of homogenizing the muscle was varied for the next set of experiments. The minced muscle fiber was homogenized using the Waring blender (2 times of 30 seconds on and 30 seconds off), the homogenate was centrifuged at 1,000 × *g*, and the supernatant was spun subsequently at 2K, 12K and 125K × *g* to yield microsomes of the first homogenization protocol.

In the second homogenization protocol, the pellet recovered from the initial low speed 1,000 × *g* spin in the first homogenization was rehomogenized using a Polytron tissumizer (4,000 rpm, 3 times of 10 secs on, with 1 minute rest intervals). The homogenate was centrifuged at 1,000 × *g* and the resulting supernatant is spun sequentially at 2K, 12K and 125K × *g* to yield microsomes of the second homogenization protocol.

In the third homogenization protocol, the pellet recovered from the initial low-speed (1,000 × *g*) spin of the second homogenization protocol was homogenized again, using a Polytron tissumizer (4000 rpm,

Table 2. Radioligand binding of crude microsomes isolated by variable homogenizations

Homogenization	Centrifugal Force (×g)	Protein (mg)	PN200-110 (pmol/mg)	Ryanodine (pmol/mg)
First	2K	228.7	0.95 ± 0.87 (217 pmol)	8.54 ± 0.09 (1953 pmol)
First	12K	87.3	3.30 ± 0.22 (288 pmol)	14.3 ± 0.07 (1248 pmol)
First	125K	125.6	1.76 ± 0.17 (221 pmol)	3.23 ± 0.24 (406 pmol)
Second	2K	57.6	7.16 ± 3.62 (412 pmol)	7.39 ± 0.39 (426 pmol)
Second	12K	71.1	28.74 ± 2.41 (2043 pmol)	19.14 ± 3.74 (1361 pmol)
Second	125K	45.2	8.60 ± 1.11 (389 pmol)	27.56 ± 1.61 (1246 pmol)
Third	2K	129	2.05 ± 0.16 (264 pmol)	4.41 ± 0.08 (569 pmol)
Third	12K	66.3	26.32 ± 2.71 (1745 pmol)	46.68 ± 3.68 (3095 pmol)
Third	125K	42.8	15.02 ± 1.87 (643 pmol)	33.30 ± 2.69 (1425 pmol)

Vesicles were isolated following successive homogenizations of the low-speed (1,000 × *g*) pellet, Radioligand binding assays and protein assays were performed as described in Materials and Methods.

3 times of 10 seconds on, with 1 minute rest intervals). Crude microsomes were isolated at three sequential centrifugal forces (2K, 12K and 125K × *g*) to yield microsomes of the third homogenization protocol.

The microsomes isolated from the first, second and third homogenization protocols were assayed for protein content, ^{3}H-PN200-110- and ^{3}H-ryanodine-binding activity.

The 12K × *g* microsomes were of particular interest because we had found in the previous centrifugation studies that the majority of triad vesicles pellet in this microsomal fraction. The binding results (Table 2) show that rehomogenizing the low-speed pellet from the first homogenization liberated additional binding sites for ^{3}H-PN200-110 and ^{3}H-ryanodine. The specific activity for ^{3}H-PN200-110 increased from 3.3 pmol/mg in the first homogenization (288 pmoles binding sites) to 28.74 pmol/mg binding activity present in the second homogenization (2043 pmoles binding sites). The specific activity for ^{3}H-ryanodine increased slightly from 14.3 pmol/mg in the first homogenization (1248 binding sites) to 19.14 pmol/mg binding in the second homogenization (1,361 binding sites). The third homogenization also increased the specific activity of the radioligand binding as well as liberated additional binding sites for ^{3}H-PN200-110 (26.32 pmol/mg or 1745 pmoles binding sites) and ^{3}H-ryanodine (46.68 pmol/mg or 3,095 pmoles binding sites).

The 2K × *g* microsomes from the first homogenization contained 228.7 mg protein but only contained 0.95 pmol/mg ^{3}H-PN200-110 binding and 8.54 pmol/mg ^{3}H-ryanodine activity. The 2K × *g* microsomes from the second homogenization contained considerably less protein (57.6 mg) and similar low specific activity for ^{3}H-PN200-110 (7.16 pmol/mg) and ^{3}H-ryanodine (7.39 pmol/mg). The 2K × *g* microsomes from the third homogenization contained 129 mg protein and very low specific activity for both ^{3}H-PN200-110 (2.05 pmol/mg) and ^{3}H-ryanodine (4.41 pmol/mg). SDS-PAGE showed that these fractions primarily contained actin and myosin along with a small number of triads, possibly entrapped by the mesh of contractile proteins.

The 125K × *g* microsomes from the first homogenization contained 125.6 mg protein and small specific activities for both ^{3}H-PN200-110 (1.76 pmol/mg) and ^{3}H-ryanodine (3.23 pmol/mg). The 125K × *g* microsomes from the second homogenization contained only a third as much protein (45.2 mg), with a greater than 4-fold increase in specific activity for ^{3}H-PN200-110 (8.6 pmol/mg). In contrast, the specific activity for ^{3}H-ryanodine increased more than 8-fold to 27.56 pmol/mg. The 125K × *g* microsomes from the third homogenization liberated about the same amount of protein as the previous homogenization (42.8 mg) with approximately a 7-fold increase in ^{3}H-PN200-110-specific activity (15.02 pmol/mg) and an 8- to 10-fold increase in ^{3}H-ryanodine-specific activity (33.3 pmol/mg) over that found in the 2K × *g* microsomes. The ^{3}H-ryanodine-specific activity in all of these fractions is greater than the ^{3}H-PN200-110-binding activity, which indicates that these fractions contain a greater percentage of uncoupled terminal cisternae than that found in the 12K × *g* microsomes.

In Fig. 1, the mean specific activity of ^{3}H-PN200-110 and ^{3}H-ryanodine binding (± standard error of the mean) are shown for isolated crude microsomes obtained at 12K × *g* (Fig. 1*A*), as well as for isolated enriched triads (Fig. 1*B*) and terminal cisternae (Fig. 1*C*) from multiple vesicle preparations (number of preparations is indicated above each bar) isolated from the first, second and third homogenizations. Enriched triads and terminal cisternae were isolated by separation on a continuous sucrose gradient, as described in Materials and Methods. ^{3}H-PN200-l10-specific activity (*white bars*) and ^{3}H-ryanodine-specific activity (*hatched bars*) are significantly higher in both the crude microsomes and the isolated triadic vesicles produced from the 2nd and 3rd homogenization of the initial 1,000 × *g* pellet (Mann–Whitney Rank Sum test, $P = <0.0001$ for paired tests between 1st and 2nd homogenizations or 1st and 3rd homogenizations).The isolated terminal cisternae separated from the crude microsomes show low ^{3}H-PN200-110

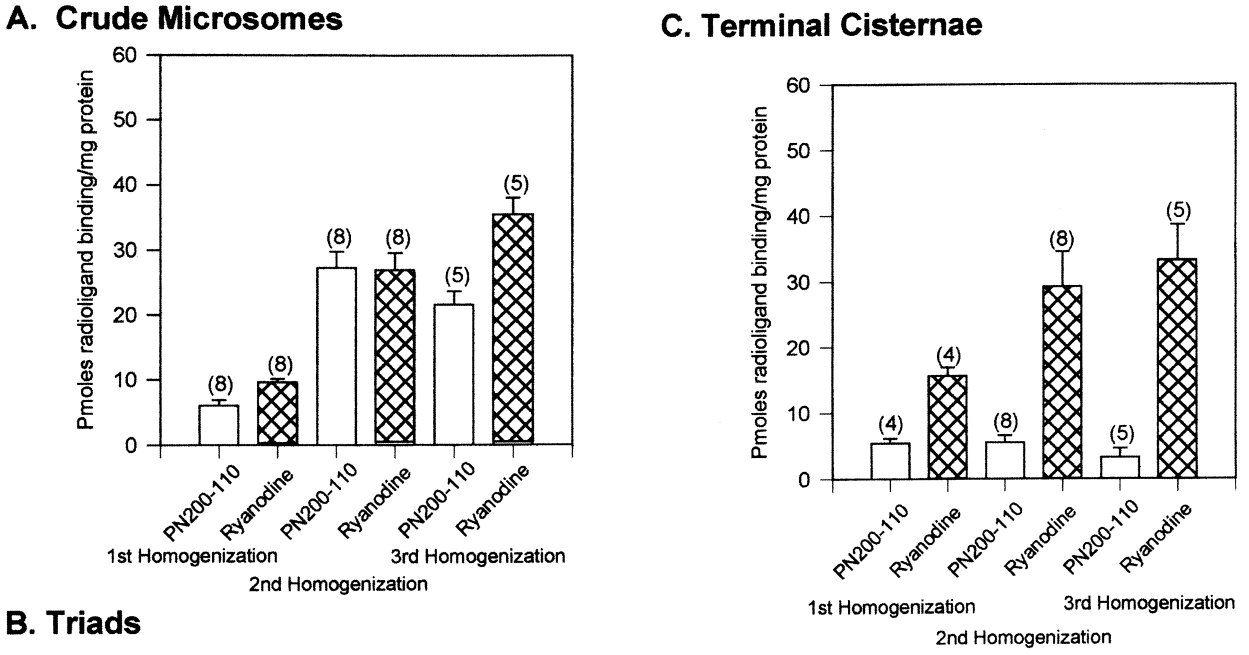

Fig. 1. Comparison of ^{3}H-PN200-110 and ^{3}H-ryanodine specific activity in crude microsomes, enriched triads and terminal cisternae isolated from different homogenizations. ^{3}H-PN200-110 (*white bars*) and ^{3}H-ryanodine (*hatched bars*) specific binding per mg protein is shown for crude microsomes (*A*), enriched triads (*B*) and enriched terminal cisternae (*C*) obtained from the 1st, 2nd and 3rd homogenizations of the 1,000 × *g* pellet. The bars represent the mean specific activities obtained from several preparations (number in parentheses above bar), ± the standard error of the mean. In *A*, the crude microsomes were obtained with a 12,000 × *g* centrifugation, following a 2,000 × *g* spin to remove contractile proteins. In *B* and *C*, the 12,000 × *g* crude microsomes were separated on continuous sucrose gradient to obtain purified triadic vesicles (*B*) and terminal cisternae (*C*).

specific activity (no significant difference between the different homogenizations) and high ^{3}H-ryanodine-specific activity, without any of the high-salt washes that are usually used to prepare terminal cisternae (Meissner, 1984). The ryanodine-specific activity in the terminal cisternae produced from the 2nd and 3rd homogenizations is significantly different from that produced in the 1st homogenization (Mann-Whitney Rank Sum test, $P = 0.018$ for paired test between 1st and 2nd homogenization and $P = 0.00088$ for paired test between 1st and 3rd homogenizations), about 3-fold greater than that normally produced for a terminal cisternae preparation (Bers & Stiffel, 1993; Hawkes et al., 1989). These data show that the preparations of both crude microsomes and the isolated enriched triads or terminal cisternae are remarkably reproducible, consistently producing enriched triads and terminal cisternae upon rehomogenization of the initial 1,000 × *g* pellet. These data indicate that triad preparations that discard the 1,000 × *g* pellet after the initial centrifugation are losing the majority of the triadic or terminal cisternae vesicles in a muscle preparation.

Although the previous data indicates that both second and third homogenizations produce vesicles with high specific activity of the dihydropyridine receptor and the ryanodine receptor, we generally preferred vesicles derived from the second homogenization for our work. We were able to successfully load these vesicles with calcium and induce depolarization-induced release with similar kinetics to that shown in our previous work (Kramer & Corbett, 1995). We could not load these vesicles with as much calcium as in our previous work (due to some loss of calsequestrin upon rehomogenization), but we saw at least 12% (routinely 12–30%) of the calcium loaded released immediately upon depolarization. The percent release obtained immediately upon depolarization varied with the coinci-

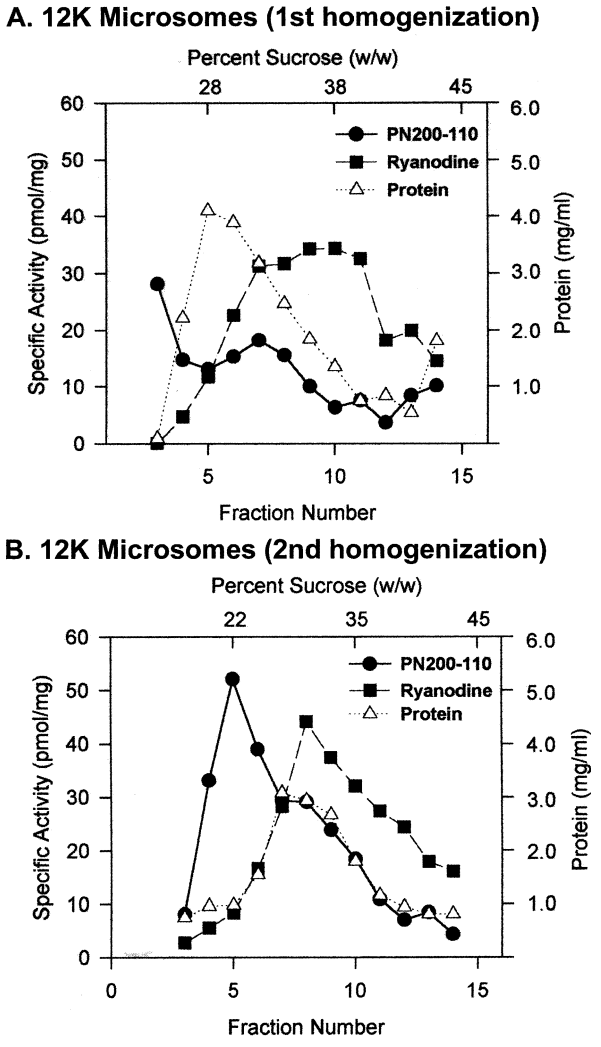

Fig. 2. Separation of crude microsomes on a continuous sucrose gradient. Crude microsomes (12,000 × *g*) were loaded onto the top of a continuous sucrose gradient from 10–45% sucrose (w/w), spun at 27,000 rpm in a SW-28 swinging bucket rotor at 5°C for approximately 16 hours. The gradients were fractionated into 2-ml fractions and assayed for ^{3}H-ryanodine and ^{3}H-PN200-110 binding, protein and percent sucrose. The separation of crude microsomes after either a single homogenization (*A*), or a 2nd homogenization (*B*) of the 1,000 × *g* pellet from *A* are shown. Specific activity of ^{3}H-PN200-110 binding is shown with the circles and solid line; specific activity of ^{3}H-ryanodine binding is shown with the squares and the dashed line. Protein concentration, using the right axis, is shown with the triangles. Coincident PN200-110 and ryanodine binding is indicative of triads. PN200-110 binding in the absence of ryanodine binding is indicative of either isolated transverse tubules or sarcolemma. Ryanodine binding in the absence of PN200-110 binding indicates isolated terminal cisternae. Typically, 2 to 4 individual fractions displaying coincident high PN200-110 and ryanodine binding were pooled (representing our enriched triad preparation) and 2 to 4 individual fractions displaying high ryanodine binding in the absence of PN200-100 binding were pooled (representing our enriched terminal cisternae preparation). The various pooled fractions from a single gradient were numbered for identity: for example, pooled fraction 2 might represent enriched triads, pooled fraction 3 might represent enriched terminal cisternae.

dent specific activity (pmoles/milligram) of the PN200-110 binding sites and the ryanodine binding sites. The vesicles from the third homogenization were typically leaky, and we were unable to successfully load them with calcium. SDS-PAGE of vesicles from the 3rd homogenization showed a reduction in calsequestrin compared to vesicles from the 2nd homogenization, which might have accounted for some of our difficulty in loading these vesicles with calcium. Vesicles from either homogenization, however, would be ideal starting material for purification of either the ryanodine receptor or the dihydropyridine receptor.

Separation of crude microsomes (12,000 × *g*) from the first and second homogenizations (Fig. 2*A* and 2*B*, respectively) on continuous sucrose gradients is shown, with protein, PN200-110 and ryanodine binding data. The specific activity of ^{3}H-PN200-110 (*circles*) and ^{3}H-ryanodine (*squares*) is shown for each 2-ml fraction from the gradient, as well as the protein concentration (right axis; *triangles*). Each fraction is also assayed for percent sucrose (w/w), shown across the top of the figure. Coincident PN200-110 and ryanodine binding is indicative of triadic vesicles: triads are found at 30–36% sucrose in Fig. 2*A* (1st homogenization) and at 28–38% in Fig. 2*B* (2nd homogenization). Isolated terminal cisternae are located at 38–45% sucrose in both homogenizations (Fig. 2*A* and 2*B*). The main difference between the two preparations is the higher specific activity of radioligand binding found in the second homogenization (Fig. 2*B*), as well as a peak of ^{3}H-PN200-110 binding at 25% sucrose found in the second homogenization. These vesicles at 25% sucrose, when examined with electron microscopy, were shown to be primarily sarcolemmal in origin (Ferguson, Lewis-Carl & Corbett, 1994).

SDS Polyacrylamide Gels

The 12K × *g* crude microsomes from the second homogenization were separated on continuous sucrose gradients as described in Materials and Methods. The gradients were fractionated and areas of interest were pooled. Figure 3*A* shows an SDS polyacrylamide gel where pooled fractions 1 through 6 isolated from the gradient were separated in lanes 1 through 6. All fractions contained a large amount of Ca-ATPase (seen at 100 kDa), the ryanodine receptor (arrowhead), and variable contamination of myosin (220 kDa), shown for a preparation before the 2,000 × *g* centrifugation was added to the protocols to remove most of this contamination. Those fractions that showed the highest specific activity of ^{3}H-PN200-110- and ^{3}H-ryanodine-binding sites (pooled fractions 3, 4, 5 in lanes 3–5) also showed the highest density of the

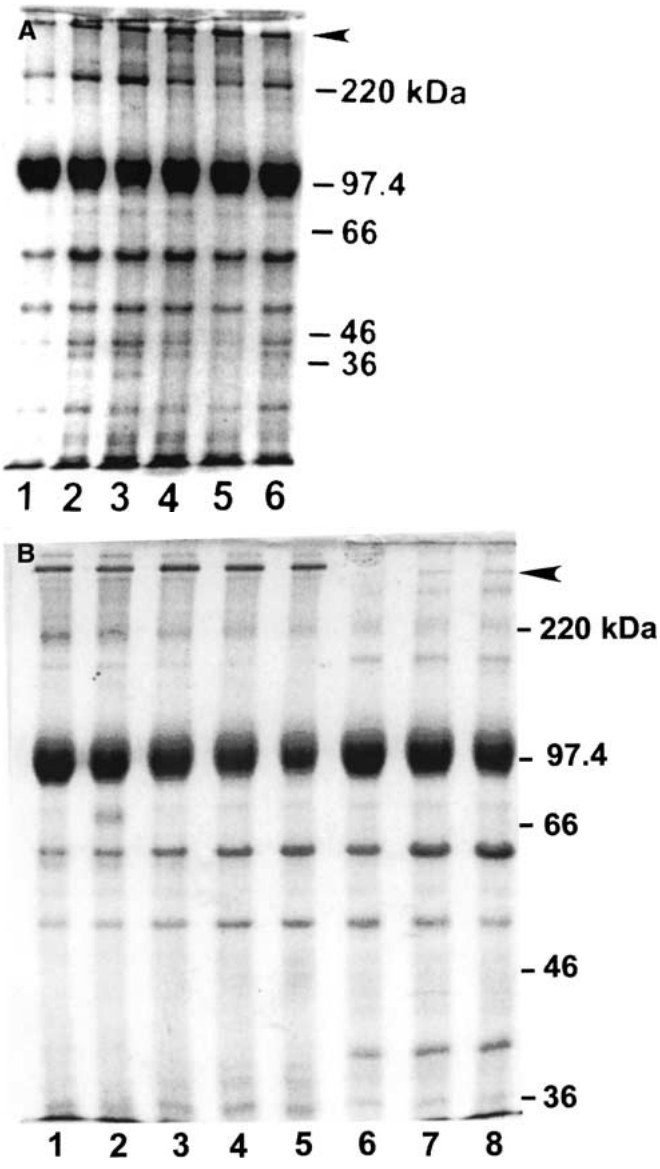

Fig. 3. SDS-PAGE of enriched triads and terminal cisternae. Crude microsomes (12,000 × *g*) were isolated using either first or second homogenizations. In (*A*) the 12,000 × *g* spin to isolate microsomes from the 2nd homogenization protocol immediately followed the 1,000 × *g* low-speed spin. In (*B*), the 12,000 ×*g* spin, to isolate microsomes from both the 1st and 2nd homogenization protocols, followed an intermediate 2,000 × *g* spin, designed to reduce contractile protein contamination. In both *A* and *B*, the crude microsomes were separated using continuous sucrose gradients. The gradients were fractionated into 2-ml aliquots and fractions of interest were pooled and loaded (20 μg/lane) onto the SDS polyacrylamide gel. In Fig. 3*A*, Lanes, *1–6* contain pooled fractions, isolated following separation of the 12,000 × *g* crude microsomes (2nd homogenization protocol using a Polytron) on a continuous sucrose gradient. Lane *1* contains vesicles from the 19–22% part of the sucrose gradient, while lane *2* was from 25%, lane *3* from 29–32%, lane *4* from 33%, lane *5* from 35–35.5%, and lane *6* was from the 37.5–40% sucrose (w/w). In Fig. 3*B*, the initial homogenization in the Waring blender was reduced to 2 × 30 pulses, and an intermediate 2,000 × *g* spin was used to reduce myosin contamination. Lanes *1–5* contain vesicles, which were isolated on sucrose gradients from crude microsomes produced by the 2nd homogenization protocol, while lanes *6–8* contained vesicles isolated on sucrose gradients from crude microsomes produced by the first Waring blender homogenization. Lane *1* contained vesicles from 28–29% on the sucrose gradient, while lane *2* was from 31–32% sucrose, lane *3* was from 33–34% sucrose, lane *4* was from 35–36% sucrose and lane *5* was from 37–43% sucrose (w/w). From the 1st homogenization protocol, vesicles in lane *6* corresponded to 35% sucrose, while lane *7* was from 36–38% sucrose and lane *8* was from 40–44% sucrose. According to radioligand binding assays, enriched triads are found in lanes *2–4* and *1–3* of the gels in *A* and *B*, respectively. Enriched terminal cisternae are found in lane *6* and lane *5* of the gels in *A* and *B*, respectively. Ryanodine receptor position is marked with an arrowhead on each gel.

ryanodine receptor. Calsequestrin (approximately 60 kDa) displayed a higher density in these vesicles, compared to vesicles isolated in fractions at lower density sucrose.

In Fig. 3*B*, pooled fractions separated from 12,000 × *g* microsomes (1st and 2nd homogenization) isolated following an intermediate 2,000 × *g* spin, to remove contamination by contractile proteins, are shown. Microsomes from the second homogenization (lanes 1–5) and the first homogenization (lanes 6–8) were separated on a continuous sucrose gradient, fractions of interest pooled according to percent sucrose, and concentrated through centrifugation. The pooled fractions were run on SDS-PAGE (Fig. 3*B*) as described in Materials and Methods. The protein profile of the pooled fractions 2–6 from the 2nd homogenization, shown in lanes 1–5, is very similar to that seen in Fig. 3*A*, except the actin and myosin contamination is reduced. Those fractions with the greatest corresponding PN200-110 and ryanodine binding activity (pooled fractions 2–4 in lanes 1–3) represent enriched triads, while pooled fraction 6 in lane 5 represents primarily enriched terminal cisternae. Pooled fractions from the 1st homogenization, which represent isolated triads (pooled fractions 1–3 in lanes 6–8), show a tremendous reduction in ryanodine channel density, corresponding well with their decreased ^{3}H-ryanodine specific activity.

Electron Microscopy

The 12K microsomes from the second homogenization were separated according to size and density on continuous sucrose gradients. Fractions from 29–32% and 38–41% sucrose were pooled and assayed for

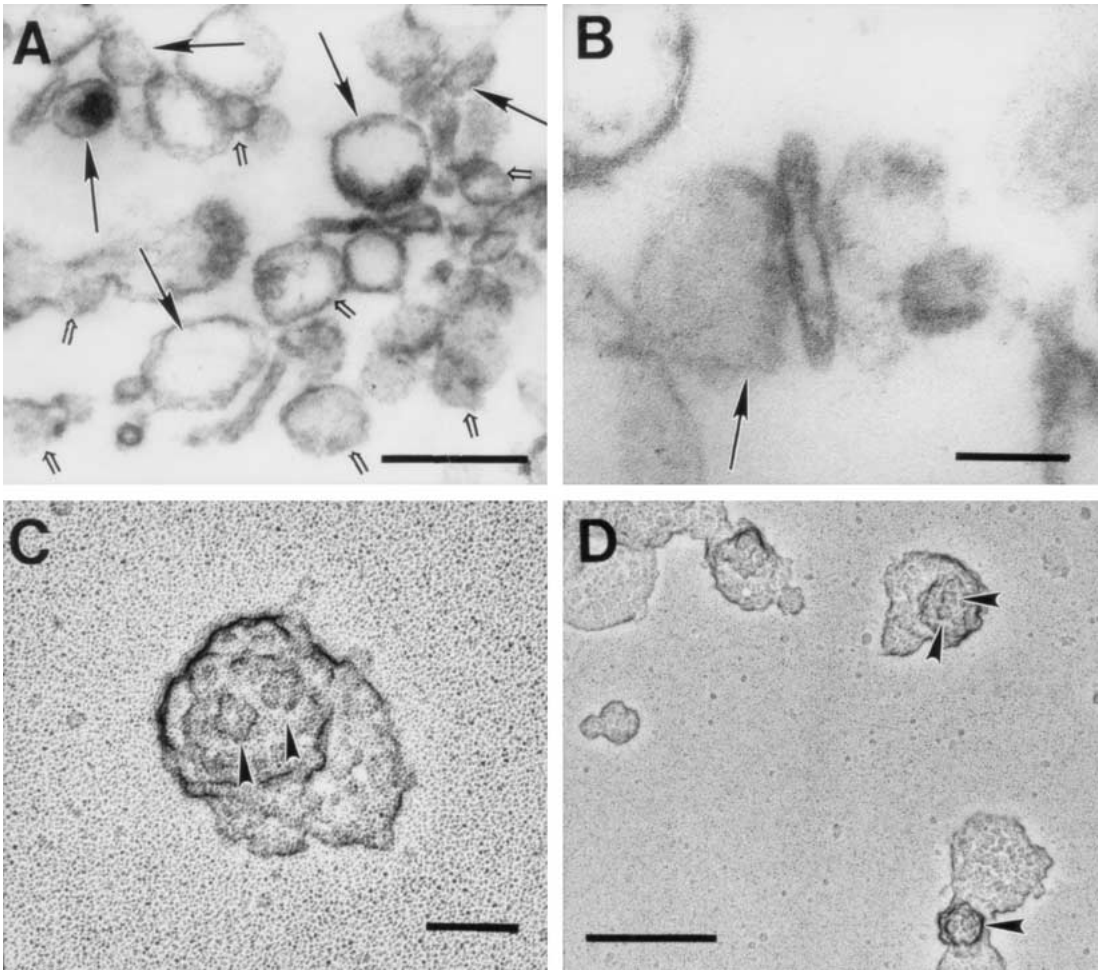

Fig. 4. Electron micrograph images of isolated triad and terminal cisternae vesicles. (*A*) Transmission electron micrograph of a thin section of isolated triadic vesicles from 12K × *g* microsomes (second homogenization protocol) separated on a continuous sucrose gradient (pooled fraction 1). Isolated terminal cisternae are indicated with an open arrow, whereas terminal cisternae that are coupled to transverse tubules (either diads or triads) are indicated with solid arrows. Scale bar = 250 nm. (*B*) Thin-section electron micrograph of isolated triad. Scale bar = 100 nm. (*C* and *D*) Rotary-shadowed freeze-dried terminal cisternae vesicles. The ryanodine receptor is indicated with arrowheads. The domed vesicles are vesicles that contain calsequestrin. Scale bars = 100 and 250 nm in *C* and *D* respectively.

protein, ^{3}H-PN200-110 and ^{3}H-ryanodine binding activity. The pooled fraction from 29–32% sucrose (pooled fraction 1) contained 7.32 mg/ml protein, which displayed high binding activity for both ^{3}H-PN200-110 (48.3 pmol/mg) and ^{3}H-ryanodine (48.6 pmol/mg). The pooled fraction from 38–41% sucrose (pooled fraction 3) contained 5.84 mg/ml protein, 22 pmoles ^{3}H-ryanodine binding/mg and lower activity for ^{3}H-PN200-110 (6.65 pmol/mg).

The pooled fraction-1 microsomes were prepared for both thin-section electron microscopy (Fig. 4*A* and 4*B*) and rotary shadowing. The thin-section micrograph clearly displays the presence of isolated terminal cisternae (TC) vesicles (*open arrows*). The solid arrows indicate terminal cisternae vesicles that are coupled to transverse tubule (TT) membranes to form both diads (1 TC + 1 TT) and triads (1 TT + 2 TC). This fraction appeared to be highly enriched in triadic vesicles. The dark smudging located inside of the terminal cisternae vesicles represents the presence of calsequestrin. Also present in the micrograph are large sheets of membrane, which may represent the sarcolemma or swollen mitochondria. An isolated triad from this fraction is shown in Fig. 4*B*.

Pooled fraction 3 vesicles were freeze-dried and rotary shadowed with platinum: these are shown in Fig. 4*C* and 4*D*. The rotary-shadowed images showed many vesicles that appeared to be dome-shaped. This appearance is characteristic of heavy sarcoplasmic reticulum vesicles that contain calsequestrin. Predominant in this fraction is the presence of calcium-release channels located in the membrane of the isolated terminal cisternae vesicles. There appeared to be a few triadic vesicles present; this correlates well with the low ^{3}H-PN200-110 binding.

Discussion

The purpose of this study was to improve the yield of triadic vesicles in a simple muscle preparation. This was accomplished by isolation procedures that increase the concurrent dihydropyridine receptor (t-tubule marker) and ryanodine receptor (terminal cisternae marker) radioligand-binding specific activity (pmoles of radioligand binding per mg protein). As a side product, we also produced an enriched preparation of isolated terminal cisternae with high yield. Many investigators use triads and/or terminal cisternae from muscle as starting material for protein purifications, particularly the dihydropyridine receptor or ryanodine receptor. By increasing the yield and specific activity of these particular binding sites, the purity of the crude material for a purification may be increased 5-fold and require a smaller number of animals to produce mg quantities of purified channels.

Two key components of the normal triad/terminal cisternae preparation were altered in order to increase triad yield and specific activity of radioligand (^{3}H-PN200-110, ^{3}H-ryanodine) binding: 1) the differential centrifugation protocol and 2) the homogenization protocol. A majority of the current protocols for triad or terminal cisternae preparation (Caswell et al., 1980; Campbell et al., 1980; Fernandez et al., 1980; Mitchell et al., 1983; Ohkusa et al., 1990; Corbett et al., 1992; Bers & Stiffel, 1993) use an initial low-speed centrifugation of 8–14,000 $\times$ g to pellet unwanted material, including contractile proteins, mitochondria and large sheets of membranes. Skeletal muscle microsomes, including triads and terminal cisternae, were only obtained with higher-speed centrifugations, ranging anywhere from 17,000 to 135,000 $\times$ g in these preparations. In the attempt to remove mitochondria from muscle preparations with an 8,000 to 14,000 $\times$ g cut, the majority of investigators have sacrificed good yields of triads and terminal cisternae. Since most investigators use millimolar concentrations of Na azide in any enzymatic assay or calcium uptake and release studies in isolated skeletal muscle vesicles to control for mitochondria contamination, this may be unnecessary waste of triadic and terminal cisternae vesicles. This study indicates that the majority (60–80%) of the ryanodine binding sites, as well as dihydropyridine receptor binding sites, are pelleted by 10,000 to 12,000 $\times$ g centrifugal force following a 1,000 and/or 2,000 $\times$ g low-speed spin to clear away contaminating material. Very high-speed centrifugations (125,000 $\times$ g) predominately pellet light, small vesicles, such as longitudinal reticulum or isolated transverse tubules (*data not shown*), and so add unnecessary contamination to a triad and/or terminal cisternae preparation. This differential centrifugation protocol used to isolate triads and terminal cisternae does not appear to be appropriate for investigators using Na pyrophosphate to homogenize the muscle (Mitchell et al., 1983; Pessah et al., 1986; Bers & Stiffel, 1992). A centrifugation protocol similar to that used in this study (Pessah et al., 1986) found that the microsomes with the highest specific activity of ^{3}H-ryanodine binding pelleted between 10,000 and 30,000 $\times$ g, with little specific activity found in microsomes pelleting between 2,000 and 10,000 $\times$ g. In these studies, the sodium pyrophosphate buffer may have shifted the sedimentation of terminal cisternae vesicles, possibly by removal of peripheral proteins as well as of actin and myosin. Investigators using buffers for homogenization of the muscle membranes other than 250 mM sucrose buffers used in these studies, then, may find it advisable to repeat the centrifugation protocols to determine the best centrifugal force at which to isolate their triads/terminal cisternae.

Since enriched triads/terminal cisternae vesicles were pelleting at such low centrifugal forces (10,000–12,000 $\times$ g), we thought it was likely that a number of triads and terminal cisternae were lost in our low-speed cut (1,000 $\times$ g) due to inefficient homogenization of the muscle. We next examined the effect of multiple homogenizations of the 1,000 $\times$ g pellet, which had previously been discarded as waste. In these studies, we determined that multiple homogenizations increased the number of ryanodine or dihydropyridine receptor binding sites, which were pelleted in the 12,000 $\times$ g microsomes, doubling or tripling the number of binding sites obtained through the first homogenization alone. The Waring blender, which is used by most investigators, is less efficient at homogenization than the Polytron tissumizer, but easier to use and less likely to clog up with fibrous material. However, when the Waring blender was used in the second and third homogenizations, markedly less (75–80% of that liberated with the Polytron) pmoles of PN200-110 and ryanodine binding sites were liberated in the 2K, 12K and 125K crude microsomes. Also, although the Polytron rehomogenization displayed the best increase in ryanodine or DHP receptor-specific activity, the terminal cisternae vesicles obtained were partially depleted of calsequestrin, inhibiting active calcium loading of the vesicles.

Rehomogenization of the 1,000 $\times$ g low-speed pellet produced a few undesirable effects, one of which was increased contractile protein contamination of the microsomes. It was necessary to add the 2,000 $\times$ g centrifugation step to our protocol to pellet the majority of the actin and myosin. As more actin and myosin were liberated, we saw that the percentage of triadic vesicles in the 2,000 $\times$ g pellet increased, rising up to 17% of the total ryanodine and/or DHP receptor binding sites contained in this pellet in second or third homogenizations. Despite this effect, we found that rehomogenizations increased the total

number of ryanodine and DHP receptor sites liberated in the 12K crude microsomes by 4- to 8-fold, indicating an increase in triad and terminal cisternae vesicle yield of at least 5-fold.

Increasing the number of homogenizations also increased the number of large sarcolemmal vesicles that were obtained from the muscle. These vesicles were generally separated at 19–25% sucrose on a continuous sucrose gradient, along with isolated transverse tubules (Ferguson et al., 1994). This may be an advantage to investigators who wish to examine sarcolemmal vesicles, but the presence of these vesicles made separation of microsomes on continuous sucrose gradients a necessity in order to obtain a pure triad/terminal cisternae preparation. Following separation on sucrose gradients, triadic vesicles typically displayed radioligand binding of 25–50 pmoles/mg for both ^{3}H-ryanodine and ^{3}H-PN200-110 and were found at 29–35% sucrose. Electron microscopy of thin sections from these fractions revealed a large number of enriched triads and diads (*see* Fig. 4). Isolated terminal cisternae (Ferguson et al., 1994; Kramer, Ritucci & Corbett, 1994) banded at 35–40% sucrose, and predominantly displayed high specific activity for ryanodine binding (30–40 pmoles/mg), while PN200-110 binding was reduced (0–10 pmoles/mg). These vesicles displayed a high concentration of ryanodine receptors, envisioned by rotary shadowing of freeze-dried samples (Fig. 4).

Anderson et al. (1994) determined that approximately 75 pmoles of both ryanodine and PN200-110 binding sites were present per gram (wet weight) of rabbit skeletal muscle. If we assume that the average wet weight of our leg muscle preparation is 400 grams, then we would expect that the total number of available binding sites would be 30 nmoles for each radioligand. If we examine the percentage of binding sites liberated in the 12K crude microsomes through our protocol, summed from all three homogenizations, then we find approximately 13% (4076 pmoles) of the available PN-200-110 sites and 19% (5704 pmoles) of the available ryanodine binding sites in this pool of crude microsomes. If we examine the percentage of binding sites liberated in a combination of both 12K and 125K crude microsomes, summed from all three homogenizations, we find approximately 17.8% (5329 pmoles) of the available PN200-110 binding sites and 29.2% (8781 pmoles) of the available ryanodine binding sites in this pool of crude microsomes. Typically, a much smaller percentage of binding sites are found in the 2K crude microsomes, summed from all three homogenization: approximately 3% of the available PN200-110 binding sites and 9.8% of the available ryanodine binding sites are in this pool of crude microsomes. This is a remarkable improvement over traditional triad/terminal cisternae preparations, in which less than 2% of the total binding sites in muscle are obtained. Further examination of the enriched triadic vesicles in this preparation may determine how many dihydropyridine receptors are coupled to each ryanodine receptor in rabbit skeletal muscle.

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