Subfractionation of cardiac sarcolemma with wheat-germ agglutinin

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The properties of highly purified bovine cardiac sarcolemma subfractionated with the lectin, wheat-germ agglutinin (WGA) were studied. Two different membrane subfractions were isolated, one which was agglutinated in the presence of 1.0 mg of WGA/mg of protein (WGA⁺ vesicles) and a second fraction which failed to agglutinate (WGA[−] vesicles). These two membrane fractions had quantitatively different rates of Na⁺/K⁺-dependent, ouabain-sensitive ATPase and Na⁺/Ca²⁺ exchange activities, yet a similar protein composition, which suggests that they were both derived from the plasma membrane. WGA[−] vesicles had a decreased number of [³H]quinuclidinyl benzilate-binding sites and no detectable [³H]nitrendipine-binding sites. Electron-microscopic and freeze-fracture analysis showed that the WGA⁺ fraction was composed of typical spherical sarcolemmal vesicles, whereas the WGA[−] fraction primarily contained elongated tubular structures suggestive of the T-tubule vesicles which were previously isolated from skeletal muscle. Assays of marker enzymes revealed that these fractions were neither sarcoplasmic reticulum nor plasma membrane from endothelial cells. Moreover, WGA agglutination did not result in the separation of right-side-out and inside-out vesicles. On the basis of these findings we propose that the WGA⁺ fraction corresponds to highly purified sarcolemma, whereas the WGA[−] fraction may be derived from T-tubule membranes.

INTRODUCTION

The plasma membrane of cardiac myocytes can be morphologically differentiated into two membranes, the sarcolemma (SL) or surface membrane and the T-tubules [1]. These membranes play an important role in regulating myocardial contractile function. The SL is involved not only in the regulation of cytosolic Ca²⁺ during excitation–contraction coupling, but also in all communication between the cell interior and the extracellular space. Several key activities that appear to be critically involved in regulating contractility in intact cardiac muscle have been identified in cardiac SL. These include muscarinic cholinceptors, β -adrenoceptors, prostaglandin receptors, angiotensin receptors, adenylate cyclase [2–6], Na⁺/K⁺-dependent ATPase [3–5], Na⁺/Ca²⁺ exchange [7], Na⁺/H⁺ exchange [8], calmodulin-dependent active Ca²⁺ transport [9] and dihydropyridine-sensitive Ca²⁺ channels [10].

The T-tubule membranes of skeletal and cardiac muscles are invaginations of the SL that may make physical contacts with the terminal cisternae of SR [1]. T-tubules are thought to be responsible for conducting the action potential to the interior of the muscle fibre where depolarization triggers Ca²⁺ release from SR. Thus, compared with SL, T-tubule membranes would be expected to contain a number of unique proteins as well as have a distinct structural organization. T-tubule

membrane vesicles from skeletal muscle have been isolated and characterized [11], and attempts have been made to purify and identify T-tubule vesicles from cardiac muscle, but only partial separation has been achieved [12,13]. Isolation efforts have been hampered by the lack of a reliable marker for the cardiac T-tubule membrane.

Previous attempts to subfractionate purified cardiac SL have utilized lectins to separate different subpopulations of membrane [14–16]. Since carbohydrate chains of membrane glycoproteins and glycolipids are extracellular, sealed right-side-out membrane vesicles should bind to lectin–Sepharose resins, whereas sealed inside-out vesicles should fail to bind. Immobilized concanavalin A (Con A) has previously been used for the fractionation of sealed cardiac SL vesicles isolated from dog [14] and rat [15]. Similarly, immobilized wheat-germ agglutinin (WGA) has been used to fractionate rabbit cardiac SL [16]. In all three studies there has been disagreement on the relative amounts of ‘leaky’, sealed inside-out and sealed right-side-out vesicles present in SL preparations.

In the present study, highly purified bovine cardiac SL vesicles isolated by the method described by Jones [17] have been subfractionated with WGA, a lectin with an affinity for *N*-acetyl- β -D-glucosamine residues. Two populations of plasma-membrane vesicles were obtained. The agglutinated vesicles represent the major proportion of the membrane vesicles, appear to have a predominantly

Abbreviations used: SL, sarcolemma; SR, sarcoplasmic reticulum; WGA, wheat-germ agglutinin; Con A, concanavalin A; PAGE, polyacrylamide-gel electrophoresis; NTP, nitrendipine; QNB, quinuclidinyl benzilate; Ca²⁺-ATPase, Ca²⁺-dependent ATPase; Na⁺/K⁺-ATPase, Na⁺/K⁺-dependent, ouabain-sensitive ATPase; WGA⁺, sarcolemmal vesicles agglutinated with wheat-germ agglutinin; WGA[−], sarcolemmal vesicles that failed to agglutinate with wheat-germ agglutinin.

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right-side-out orientation and correspond to highly purified SL. The non-agglutinated vesicles contain plasma-membrane marker-enzyme activities and reduced numbers of [^3H]quinuclidinyl benzilate ([^3H]QNB)- and [^3H]nitrendipine ([^3H]NTP)-binding sites compared with agglutinated vesicles. Their ultrastructure suggests that they may be derived from T-tubule membranes.

EXPERIMENTAL

Materials

[^3H]Ryanodine, [^3H]NTP, [^3H]QNB, and $^{45}\text{CaCl}_2$ were obtained from New England Nuclear. ATP, histidine, Tris, WGA, biotinylated WGA, biotinylated Con A, Arsenazo III, alamethicin, ouabain, digitoxigenin, valinomycin, atropine and *N*-acetyl-D-glucosamine were purchased from Sigma. Pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase, NAD^+ and NADH were obtained from Boehringer Mannheim. SDS/polyacrylamide-gel electrophoresis (PAGE) reagents and molecular-mass markers were purchased from Bio-Rad. Vecta stain ABC was purchased from Dimension Laboratories. Fresh calf hearts were obtained from a local slaughterhouse. Nifedipine was a gift from Miles Laboratories.

Preparation of SL vesicles

SL membranes were isolated by the sucrose-flotation method of Jones [17]. Vesicles were suspended at a concentration of 5–10 mg/ml in 250 mM-sucrose/10 mM-histidine, pH 7.5, frozen in liquid N_2 and stored at -85°C for not more than 3 months before use. Before use, samples of frozen membrane were thawed rapidly at 37°C . Thawed samples of membranes showed identical Na^+/K^+ -dependent, ouabain-sensitive ATPase (Na^+/K^+ -ATPase) and $\text{Na}^+/\text{Ca}^{2+}$ exchange activities and sensitivity to inhibitors as freshly prepared material. Protein was determined by the method of Peterson [18].

WGA agglutination of SL vesicles

An aliquot of SL membrane was diluted to 1 mg of protein/ml and then mixed with an equal volume of WGA (1 mg of protein/ml) in 160 mM-NaCl/10 mM-sodium phosphate, pH 6.8. Unless otherwise specified in the text, the ratio of WGA to SL was 1:1 (w/w). The mixture was incubated on ice for 10 min and centrifuged for 1 min in an Eppendorff centrifuge at 14000 *g*. The pellet contained lectin-agglutinated SL vesicles (WGA^+ vesicles). Agglutinated membrane vesicles were washed twice with 1 ml aliquots of 250 mM-sucrose/10 mM-histidine, pH 7.5. The supernatant was removed and centrifuged (104000 *g*) for 5 min at 4°C in a Beckman Airfuge at an air pressure of 206.7 kPa. The pellet from the high-speed centrifugation contained non-agglutinated SL vesicles (WGA^- vesicles). Both WGA^+ and WGA^- vesicles were resuspended in 250 mM-sucrose/10 mM-histidine, pH 7.5, then used for analysis after protein determination by the method of Peterson [18]. WGA^+ membrane vesicles were also routinely deagglutinated with 200 mM-*N*-acetyl-D-glucosamine in 250 mM-sucrose/10 mM-histidine, pH 7.5. As deagglutinated WGA^+ vesicles had biochemical and pharmacological characteristics similar to those of WGA^+ vesicles (results not shown), only results obtained with WGA^+ and WGA^- vesicles are presented here.

ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchange assays

Na^+/K^+ -dependent ATPase activity was measured in medium consisting of 100 mM-NaCl, 10 mM-KCl, 0.2 mM-phosphoenolpyruvate, 3 mM- MgCl_2 , 3 mM-ATP, 0.2 mM-NADH, 8.75 units of pyruvate kinase/ml, 12.5 units of lactate dehydrogenase/ml and 50 mM-histidine, pH 7.0, in the presence or absence of 1 mM-ouabain. The change in absorbance at 340 nm due to NADH oxidation was measured at 37°C with Shimadzu UV-3000 spectrophotometer. Membrane vesicles were pretreated for 30 min with 1 mg of alamethicin/mg of protein to permit the determination of total ATPase activity [5]. Ca^{2+} -dependent ATPase activity was assayed as described previously [19]. Na^+ -dependent Ca^{2+} uptake ($\text{Na}^+/\text{Ca}^{2+}$ exchange activity) was measured by using the dye Arsenazo III as described previously [20]. The rates of Na^+ -dependent Ca^{2+} uptake were calculated as the difference between rates of Ca^{2+} uptake occurring in media containing 160 mM-KCl and 160 mM-NaCl.

Electron microscopy

Thin-section electron microscopy of SL vesicles was carried out as described previously [19]. For freeze-fracture analysis, membrane vesicles were pelleted in a Beckman Airfuge (air pressure 206.7 kPa) and fixed in 4% (w/v) glutaraldehyde in 100 mM-phosphate buffer, pH 7.4. This was followed by incubation in 30% (v/v) glycerol, rapid freezing in Freon 22, fracturing and shadowing in a Balzers BAF 080 apparatus. A total of three independent membrane preparations were used for the thin-section and freeze-fracture electron microscopy analysis. Pelleted membrane vesicles were either sectioned or fractured across the pellets (top to bottom). A total of 20 profiles were examined for every thin section or freeze-fracture experiment. Typical electron micrographs of SL, WGA^+ and WGA^- vesicles are presented in Figs. 2 and 3 (below).

Binding of ^3H -labelled ligands to membrane vesicles

[^3H]QNB binding was measured as described by Manalan *et al.* [21]. A 50 μg portion of membrane protein was incubated at 22°C in 1 ml of 1 nM-[^3H]QNB/250 mM-sucrose/9 mM- MgCl_2 /1 mM-ascorbic acid/50 mM-Tris, pH 7.5, in the presence or absence of 1 μM -atropine. Two 450 μl aliquots of incubation mixture were transferred to Whatman GF/C filters and rinsed three times with 5 ml washes of ice-cold 9 mM- MgCl_2 /50 mM-Tris, pH 7.5. Specific binding was calculated as the difference between the amount of ligand bound in the presence or absence of atropine. [^3H]NTP binding assays were conducted as described by Howlett & Gordon [22]. Briefly, 12 μg of membrane protein was incubated for 90 min at 22°C in 1 ml of 50 mM-Tris, pH 7.4, containing 0.013–0.650 nM-[^3H]NTP. Assays were terminated by filtering sample through Whatman GF/B filters and washing six times with 2 ml of ice-cold Tris buffer using a Brandel cell harvester (model 24RC; Brandel, Gaithersburg, MD, U.S.A.). Specific binding was calculated as the difference between the amount of [^3H]NTP bound in the presence or absence of 20 nM unlabelled nifedipine. [^3H]Ryanodine binding to membrane vesicles was carried out at 37°C for 120 min in a solution containing 20 nM-[^3H]ryanodine as described previously [23]. All data are presented as the mean $\pm$ S.E.M.

Fig. 1. Lectin agglutination of SL vesicles

Portions (100 μ l) of SL membrane (1 mg of protein/ml) were agglutinated with different amounts of WGA as described in the Experimental section. Agglutinated membrane vesicles were centrifuged at 14000 g for 10 min and the protein content was determined in the pellet (agglutinated vesicles, ■) and supernatant (non-agglutinated vesicles, □). At 0.5 mg of WGA/mg of SL protein, approx. 75% of the membrane was agglutinated.

SDS/PAGE

SDS/PAGE was performed with either 10 or 5–15% (w/v) polyacrylamide gradient gels using the discontinuous buffer system of Laemmli [24]. Gels were stained with silver, Coomassie Blue or Stains-All [25]. Molecular-mass standards were: myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 42 kDa; and bovine carbonic anhydrase, 31 kDa.

Analysis of lectin and $^{45}\text{Ca}^{2+}$ binding to membrane proteins

For lectin and $^{45}\text{Ca}^{2+}$ binding analysis, membrane proteins transferred to nitrocellulose [26] were first incubated with $^{45}\text{Ca}^{2+}$ by the method of Maruyama *et al.* [27], and, subsequently, the same transfers were blocked with 0.3% gelatin (in 10% ethanolamine)/100 mM-Tris, pH 9.0, and incubated with biotinylated lectins (1 μ g/ml) in a buffer containing 0.25% gelatin, 150 mM-NaCl, 0.05% Nonidet P40, 5 mM-EDTA and 50 mM-Tris,

Table 1. Enzymic activities of SL subfractions

The specific activities of the Na^+/K^+ -dependent ATPase and the $\text{Na}^+/\text{Ca}^{2+}$ exchange were determined as described in the Experimental section. The number in parentheses denotes the number of preparations analysed.

Fraction	Na^+/K^+ -dependent, ouabain-sensitive ATPase (μ mol of P_i /h per mg of protein)	$\text{Na}^+/\text{Ca}^{2+}$ exchange (nmol of Ca^{2+} /5 s per mg of protein)
SL	126 $\pm$ 18 (3)	42 $\pm$ 3 (3)
WGA ⁺	108 $\pm$ 30 (3)	40 $\pm$ 5 (3)
WGA ⁻	40 $\pm$ 6 (3)	25 $\pm$ 5 (3)

pH 7.4 [28]. Blots were washed and then incubated with Vectastain ABC and subsequently developed with 3,3'-diaminobenzidine/0.03% H_2O_2 /50 mM-Tris, pH 7.5.

RESULTS

Lectin agglutination of SL vesicles

Fig. 1 shows the WGA-concentration-dependence of SL agglutination. Approx. 75% of SL vesicles could be maximally agglutinated by concentrations of WGA in excess of 0.5 mg/mg of protein (Fig. 1). Thus, in the experiments presented in this paper, SL vesicles were

Fig. 2. Thin-section electron micrographs of SL (a), WGA⁺ (b) and WGA⁻ (c and d) vesicles

SL contains a mixture of vesicular and elongated profiles (a). Vesicular profiles are enriched in the WGA⁺ fraction (b) and elongated tubular structures are enriched in the WGA⁻ fraction (c). Electron-dense material can be seen at the ends of some of the tubular structures in WGA⁻ (d). The bar represents 0.5 μ m.

Fig. 3. Freeze-fracture electron micrographs of WGA⁺ (a) and WGA⁻ (b and c) vesicles

WGA⁺ vesicles appear as spherical membrane vesicles (a), whereas the WGA⁻ vesicles appear as elongated tubular and 'sheet-like' membrane structures (b), some of which show disc-shaped membrane morphology (c). The bar represents 1.0 μm .

agglutinated with WGA at a ratio of 1 mg of WGA/mg of SL protein.

Enzymic activities in SL subfractions

Plasma-membrane enzyme markers were detected in

all SL subfractions analysed. Table 1 shows Na⁺/K⁺-dependent ATPase and Na⁺/Ca²⁺ exchange activities in SL subfractions after WGA agglutination. Compared with SL and WGA⁺, the WGA⁻ vesicles had reduced Na⁺/K⁺-dependent ATPase and lower Na⁺/Ca²⁺ ex-

Fig. 4. Protein pattern of SL vesicles subfractionated by lectin agglutination

Membrane vesicles were subjected to SDS/PAGE (5–15 % polyacrylamide gradient gels) as described in the Experimental section. A 30 μ g portion of membrane protein was loaded on to each lane and the gel was stained with silver. Lane 1, SL vesicles; lane 2, WGA⁺ vesicles; lane 3, WGA⁻ vesicles.

change activities (Table 1). The activities of these enzymic markers in SL and WGA⁺ vesicles, however, were not significantly different (Table 1). All three fractions exhibited a very low activity of Ca²⁺-ATPase and very low rates of ATP-dependent Ca²⁺ transport (< 4 nmol of Ca²⁺/min per mg of protein).

The sidedness of the SL, WGA⁺ and WGA⁻ vesicles was estimated from the ouabain and digitoxigenin inactivation of Na⁺/K⁺-ATPase activity in the presence or absence of alamethicin [5,29]. The SL vesicles used in this work were routinely composed of 30 % 'leaky' and 70 % right-side-out sealed vesicles. Both the WGA⁺ and WGA⁻ fractions contained approx. 50 % leaky and 50 % right-side-out vesicles. These results showed the WGA agglutination of SL vesicles did not lead to separation of two differently oriented subpopulations of membrane vesicles, i.e. inside-out vesicles and right-side-out vesicles.

Electron-microscopic observations of membrane vesicles

Fig. 2 shows survey electron micrographs of thin-

Fig. 5. Lectin and ⁴⁵Ca²⁺ binding to SL subfractions

A 30 μ g portion of membrane protein was separated by SDS/PAGE and transferred to nitrocellulose membranes as described in the Experimental section. Blots were allowed to react with WGA (a), Con A (b) and ⁴⁵Ca²⁺ (c) as described in the Experimental section. 1, SL vesicles; 2, WGA⁺ vesicles; 3, WGA⁻ vesicles. The same membrane preparation was used in (a), (b) and (c).

sectioned pellets taken from SL, WGA⁺ and WGA⁻ vesicles. SL was ultrastructurally heterogeneous and contained a mixture of vesicular and elongated profiles (Fig. 2a). Agglutination of SL membranes led to the enrichment of vesicular profiles in the WGA⁺ fraction (Fig. 2b). Elongated tubular structures were enriched in the WGA⁻ fraction (Fig. 2c). Some of the tubular structures in the WGA⁻ fraction contain electron-dense material at the ends (Fig. 2d). These structural differences between WGA⁺ and WGA⁻ vesicles are dramatically illustrated in Fig. 3. WGA⁺ vesicles appeared in freeze-fracture as typical spherical membrane vesicles (Fig. 3a). The WGA⁻ fraction, however, contained a large number of elongated tubular and 'sheet-like' membrane structures (Fig. 3b), some of which show discus-shaped membrane morphology (Fig. 3c).

Protein pattern of SL subfractions

Fig. 4 shows the SDS/PAGE protein profiles of SL vesicles fractionated with WGA. Although WGA⁺ and WGA⁻ fractions differed substantially with respect to morphology (Figs. 2 and 3), they had similar polypeptide compositions (Fig. 4). The WGA⁺ and WGA⁻ fractions did, however, differ slightly in that the WGA⁻ fraction was characterized by increased silver staining of 100 and 90 kDa protein bands and reduced staining of >400, 125, 110, 95 and 66 kDa protein bands (compare lane 3 with lanes 1 and 2; Fig. 4). This was confirmed by densitometric scans of Coomassie Blue-stained gels (results not shown). Staining of the SDS/PAGE gels with Stains-All, which detects calsequestrin [25], revealed no blue-stained protein bands (results not shown).

Lectin and ⁴⁵Ca²⁺ binding to membrane proteins

Membrane glycoproteins may be identified by lectin binding to proteins electrophoretically transferred to nitrocellulose membranes [30]. Fig. 5(a) shows WGA binding to membrane proteins of approx. 120 kDa and 100 kDa present in SL vesicles. Some WGA binding was also observed to 200, 70 and 55 kDa glycoproteins (Fig.

Fig. 6. NTP binding to SL subfractions

[³H]NTP binding was carried out as described in the Experimental section. ▲, Total binding; △, non-specific binding in the presence of 20 nM-nifedipine, ■, specific binding (total binding minus non-specific binding). Abbreviations: SL, sarcolemmal vesicles; WGA⁺, agglutinated membrane vesicles; WGA⁻, non-agglutinated membrane. Data are from a typical experiment.

5a, lane 1). The same WGA binding pattern was observed in WGA⁺ vesicles (Fig. 5a, lane 2). The WGA⁻ fraction, however, bound only small amounts of lectin to a 120 kDa glycoprotein (Fig. 5a, lane 3). Con A binding to membrane proteins is shown in Fig. 5(b); the Con A-binding glycoproteins in SL subfractions had molecular masses of 110, 100, 70, 55, 45, 40 and 30 kDa (Fig. 5b). In SL and WGA⁺ fractions the major Con A-reactive glycoprotein band had a molecular mass of 100 kDa (Fig. 5b, lanes 1 and 2), but in WGA⁻ vesicles it was a 110 kDa protein (Fig. 5b, lane 3).

Fig. 5(c) shows ⁴⁵Ca²⁺ binding to membrane proteins analysed by the ⁴⁵Ca²⁺ overlay technique [27]. The 120 and 100 kDa WGA-binding glycoproteins of SL and WGA⁺ fractions were also the major Ca²⁺-binding proteins (Fig. 5c). There was, however, reduced ⁴⁵Ca²⁺ binding to these proteins in WGA⁻ vesicles relative to SL and WGA⁺ fractions (Fig. 5c, compare lane 3 with lanes 1 and 2).

Ligand binding

[³H]QNB binding, a measure of the number of muscarinic receptors, was low in WGA⁻ [0.8 ± 0.1 (3) pmol/mg of protein] when compared with the other two fractions [3.0 ± 0.1 (3) pmol/mg of protein in SL and 3.0 ± 0.2 (3) pmol/mg of protein in WGA⁺ vesicles]. [³H]Ryanodine was used as a sarcoplasmic reticulum (SR) marker; no binding was detected in any of the three fractions tested. Fig. 6 shows the binding of [³H]NTP to SL subfractions. High specific binding of [³H]NTP

was observed in SL and WGA⁺ vesicles (for WGA⁺, $K_d = 0.14 \pm 0.04$ nM, $n = 4$; $B_{max} = 0.7 \pm 0.2$ pmol/mg of protein, $n = 4$; values for unfractionated SL vesicles were similar). In contrast, WGA⁻ vesicles showed no specific [³H]NTP binding, although a small amount of non-specific binding was observed (Fig. 6).

DISCUSSION

Two distinct membrane populations were separated by WGA agglutination of highly purified SL. Approx. 75% of SL vesicles could be maximally agglutinated by concentrations of WGA in excess of 0.5 mg/mg of protein and, despite repeated additions of WGA, 25% of the SL vesicles failed to agglutinate. Plasma-membrane enzyme markers, such as Na⁺/K⁺-ATPase and Na⁺/Ca²⁺ exchange, were detected in all three subfractions (SL, WGA⁺ and WGA⁻), which confirms that both WGA⁺ and WGA⁻ vesicles were derived from plasma membrane. This is also supported by the similar polypeptide composition of all three fractions.

One logical explanation for the separation of WGA⁺ and WGA⁻ vesicles by lectin agglutination is that WGA⁺ vesicles represent right-side-out SL vesicles, whereas the WGA⁻ vesicles represent inside-out vesicles. Previous attempts to subfractionate cardiac SL vesicles have employed lectin chromatography to separate sealed right-side-out and inside-out membrane vesicles [14–16]. These experiments were based on the assumption that inside-out vesicles should fail to interact with WGA or Con A and have increased specific activities of enzyme markers which have cytoplasmically oriented substrate binding and catalytic sites (e.g. Na⁺/K⁺-ATPase, adenylate cyclase). By contrast, right-side-out vesicles should bind to lectin affinity columns and, when eluted with an excess of competitive sugar, should show enrichment of receptor-binding sites (e.g. muscarinic, acetylcholine, adrenergic). On the basis of measurements of Na⁺/K⁺-ATPase and the ability of alamethicin to unmask latent enzyme activity or ouabain-binding sites, we concluded that the bovine cardiac SL isolated in this study by the method of Jones [17] consisted primarily of right-side-out and some 'leaky' membrane vesicles. Receptor binding studies also confirmed these observations. Both WGA⁺ and WGA⁻ vesicle fractions, however, consisted of an equal proportion of right-side-out and 'leaky' vesicles. Therefore WGA agglutination of SL vesicles did not lead to the separation of right-side-out and inside-out vesicles. On the basis of their biochemical, morphological and pharmacological properties, we conclude that the WGA⁺ vesicles represent highly purified cardiac SL membranes. The origin of the WGA⁻ fraction is less clear.

We have considered the possibility that WGA⁻ vesicles may arise from other membrane systems present in cardiac muscle. [³H]Ryanodine, which binds to the Ca²⁺-release channel in the SR [29], was used as an SR marker, and no binding was detected in any of the three fractions tested. The degree of contamination of SR in SL fractions was also determined with Ca²⁺-ATPase assays. All three fractions exhibited very low activity of SR Ca²⁺-ATPase and very low rates of ATP-dependent Ca²⁺ uptake. Considerable activity of Ca²⁺-ATPase was detected, however, in the membrane fractions banded at the 0.6/1.0 M interface of the sucrose flotation gradient [17], indicating that a very good separation of SL and SR

membrane vesicles was achieved. The degree of SR contamination of SL fractions was also assessed by staining SDS/PAGE gels with Stains-All to detect cal-sequestrin [25]. No blue-stained protein bands were detected in any of the three SL subfractions (results not shown). The results of these experiments indicate that the SL fractions used in these studies were not contaminated with SR membranes.

We also considered that WGA⁻ vesicles may have been derived from non-muscle plasma membranes. Angiotensin-converting-enzyme assays have been used as markers of endothelial-cell plasma membrane, and some activity was detected in purified SL vesicles [31]. In the present study there was no detectable activity of angiotensin-converting enzyme in the WGA⁻ fraction (M. Rabinovitch, unpublished work), ruling out the possibility that WGA⁻ vesicles were derived from endothelial plasma membrane.

Another possible origin of the two different SL subfractions is that they were derived from functionally different regions of the cardiac myocyte plasma membrane. SL forms two membrane systems in cardiac and skeletal muscle, the surface membrane and T-tubule membrane. Although cardiac T-tubule vesicles have not been isolated, the T-tubule membrane has been isolated and characterized in skeletal muscle. Skeletal-muscle T-tubules are characterized by an elongated tubular appearance, a high number of binding sites for [³H]NTP and relatively high Mg²⁺-ATPase activity, tentatively identified as a Con A-binding protein of approx. 100 kDa [32–35]. These markers have been used extensively for the skeletal-muscle T-tubule membrane; however, to date no good biochemical marker for the cardiac T-tubule has been described. Some of our morphological and biochemical observations suggest that the WGA⁻ vesicles resemble the T-tubule vesicles of skeletal muscle.

The following observations support our hypothesis that WGA⁻ vesicles may be derived from the cardiac T-tubule membrane. Most WGA⁻ vesicles appear as elongated, tubular structures. Both thin-section and freeze-fracture profiles of the WGA⁻ vesicles resemble those observed for the T-tubule vesicles isolated from skeletal muscle [33,34]. As in the skeletal-muscle T-tubule, a Con A-binding protein of approx. 100 kDa was identified in WGA⁻ vesicles. [³H]QNB binding, a measure of the number of muscarinic receptors, was low in WGA⁻ vesicles compared with the other two fractions. [³H]QNB binding would be expected to be low in T-tubule membranes, as nerve endings do not penetrate down the T-tubule in cardiac muscle. In the heart, muscarinic receptors would be expected to be localized in the surface membrane, not in the T-tubules.

The WGA⁻ fraction showed no specific [³H]NTP binding. If the WGA⁻ fraction is indeed derived from cardiac T-tubule membrane, it is surprising that it does not contain any [³H]NTP-binding sites. In cardiac skeletal muscle, T-tubule membranes consist of junctional (interacting with terminal cisternae of SR) and non-junctional T-tubules having different morphological features [1]. Although [³H]NTP binding is the best marker for the skeletal-muscle T-tubule membranes [35], low numbers of dihydropyridine-binding sites were reported in non-junctional T-tubules isolated from skeletal muscle [36]. Thus, if the WGA⁻ vesicles isolated in the present study are derived from T-tubule membranes, they are more

likely to correspond to secondary or non-junctional T-tubule membranes.

In conclusion, using a WGA agglutination technique we have separated two distinct membrane populations from highly purified SL. The WGA⁺ fraction contained vesicles corresponding to highly purified SL membranes. The non-agglutinated WGA⁻ vesicles may correspond to the non-junctional T-tubule membranes.

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