Ultrastructure of Isolated Sarcolemma from Dog and Rabbit Myocardium
Comparison to Intact Tissue

J.S. Frank, K.D. Philipson, and S. Beydler

From the Departments of Medicine and Physiology and the American Heart Association Greater Los Angeles Affiliate Cardiovascular Research Laboratories, University of California, Los Angeles, School of Medicine, Center for the Health Sciences, Los Angeles, California

SUMMARY. We compared the morphology of cardiac sarcolemmal membranes isolated from dog and rabbit hearts with the sarcolemma in intact cells, using freeze-fracture and thin-section electron microscopy. In addition, we estimated the sidedness of the isolated sarcolemma based on its freeze-fracture morphology and biochemical determinations of sialic acid content and Na,K-ATPase activity. The bilayer in isolated membranes is similar, in its morphology, to intact membrane. The isolated sarcolemmal vesicles have the same density of intramembrane particles per \( \mu m^2 \) as the intact sarcolemma (\( \sim 2800/\mu m^2 \)). The particle counts in isolated sarcolemma were very homogeneous, with the peak in particle density curve the same as found for intact cells. In the intact myocardium, both sarcolemmal and transverse tubular membranes have the same density of intramembrane particles. Thin-section morphology of the isolated sarcolemma shows an intact surface coat, whereas portions of the external lamina are absent. Both the biochemical and morphological data indicate that there is a substantial fraction of inside-out and right side-out vesicles in this preparation. Considering the approximations inherent in both morphological and biochemical approaches, we find the qualitative agreement of the estimations of vesicle orientation noteworthy. (Circ Res 54: 414-423, 1984)

ISOLATED and purified cardiac sarcolemmal (SL) membranes have been used to identify and characterize Na-Ca exchange (Reeves and Sutko, 1979; Philipson and Nishimoto, 1982) as well as ATP-dependent Na (Philipson and Nishimoto, 1983) and Ca pump (Caroni and Carafoli, 1980) activities in the heart. Given the importance of this type of preparation in unraveling cationic exchange across the SL, it is important to know how the structure of isolated, vesiculated SL compares to the intact membrane. To this end, we used isolated SL membrane and compared its morphology with that of the intact SL using freeze-fracture and thin-section electron microscopy.

Specifically, we used Weibels' stereological methods (Weibel et al., 1976) (freeze-fracture stereology), where one counts particles (IMPs) inside a test area and computes an IMP density. IMP densities were first determined in intact tissue for SL, transverse tubular (TT) system and sarcoplasmic reticular (SR) membranes. This allowed for a quantitative comparison of the freeze-fracture morphology of intact membranes critical to cardiac excitation-contraction coupling. In addition, it served as a guide with which to compare the IMP density of isolated SL. With these techniques, we estimated the degree to which the isolated vesicles are oriented right side-out vs. inside-out, and verified the high degree of homogeneity of the SL preparation.

Methods

Intact Tissue

Tissue samples were taken from dog (\( n = 2 \)) and rabbit (\( n = 2 \)) hearts that were used for isolating SL membranes. Small samples (\( \leq 0.5 \text{ mm} \)) were taken from papillary muscle and both ventricles. The tissue was fixed by immersion in 2.0% buffered (0.1 M Na cacodylate) glutaraldehyde (pH 7.4) for 2 hours, then rinsed in buffer, and gradually infiltrated with 25% glycerol. The tissue was kept in glycerol for 1 hour before processing for freeze-fracture.

To verify that immersion fixation does not distort the morphology, a series of rabbit hearts were fixed by perfusion as previously described (Frank et al., 1980, 1982). The freeze-fracture data from this tissue was obtained in the same manner as from the immersion fixed tissue. The freeze-fracture morphology did not differ between perfusion and immersion fixation.

Sarcolemmal Isolation

We isolated vesicles from trimmed canine ventricles by modifying a previously published procedure (Philipson and Nishimoto, 1982). Minced ventricles (50-100 g) were homogenized (Waring Blender, 2 \times 10 \text{ seconds}, high speed) at 4°C in 250 mm sucrose, 100 mm KCl, 25 mm Na pyrophosphate, 1 mm dithiothreitol, and 20 mm Tris/malate (pH 7.6, 22°C). The homogenate (320 ml) was then centrifuged at 48,000 g for 35 minutes. The pellet was suspended in the same medium and centrifuged again. The sides of the centrifuge tubes were then wiped dry, and the pellets (except for the small, firm, dark-brown
portion at the bottom of the tube) were suspended with a glass-Teflon homogenizer in 220 ml of 250 mM sucrose, 1 mM dithiothreitol, and 20 mM Tris/maleate (pH 7.6 at 22°C). DNase I (135,000 Kunitz units; Sigma DN-100) was added, and the preparation was incubated, with stirring, for 45 minutes at 30°C. The suspension then was disrupted with a Polytron (PT 20; 5 × 7 seconds at a setting of 5.7) and centrifuged at 13,000 g for 15 minutes. The supernatant was carefully removed and centrifuged at 160,000 g for 45 minutes. The resulting supernatant was discarded, the tubes wiped dry, and the pellets were resuspended in 24 ml of 48% sucrose (wt/wt), 0.2 mM NaCl. This suspension then was layered on the bottom of discontinuous sucrose gradients (4 ml/gradient) containing 34, 32, 29, 26, and 11% (4, 4, 5, 7, and 4 ml, respectively) sucrose (wt/wt). The 34, 32, and 29% sucrose layers also contained 0.2 mM NaCl, and the 26 and 11% layers contained 0.4 mM NaCl and 0.1 mM EGTA. The gradients were spun overnight (~16 hours) at 110,000 g in a Beckman SW 27 rotor. A white band at the top of the 26% sucrose was collected as the SL fraction. The SL was diluted, spun down (160,000 g, 75 minutes), and resuspended in either 140 mM NaCl or KCl, 10 mM MOPS (pH 7.4, 37°C), and stored in liquid nitrogen in small aliquots.

The inclusion of low [NaCl] in the sucrose gradient is an important modification. This was discovered after switching to a more highly purified water (Gelman, Water-Filer System). Omitting the NaCl from the overnight sucrose gradients that are prepared with the purer water results in SL vesicles with decreased (by ~50%) Na-Ca exchange activity and decreased ion permeability barriers. Apparently, trace ions in our former less pure water source were serving this role. The EGTA is included in the sucrose gradient to lower the endogenous Ca++ level in the final SL preparation, but it has no apparent effects on the properties of the isolated SL. Another modification from our previously published procedure (Philipson and Nishimoto, 1982) is that we now include 100 mM KCl instead of 300 mM KCl in the initial homogenization steps. This gives a moderate decrease in the number of leaky vesicles in our preparation, as detected by Na-K-ATPase measurements (see below). In three recent SL preparations using canine ventricles, the activity of the SL marker 3-dephosphorylation of ATP by KG. The reaction was carried out at 37°C for 15 minutes, using about 10 µg SL protein in a final volume of 0.1 ml. Inorganic phosphate was assayed by the method of Fiske and SubbaRow (1925).

### Sialic Acid Determinations

 Vesicles were first spun down and resuspended in 10 mM Tris/maleate (pH 6.0, 37°C). To determine total sialic acid content, aliquots were added to an equal volume of 0.2 M H2SO4 and incubated at 80°C for 1 hour. To determine how much sialic acid could be released by neuraminidase, aliquots (0.06 ml, ~3 mg/ml) were incubated with neuraminidase (Sigma, type X; 0.2 U) for 30 minutes at 37°C. This was done in the presence and absence of Triton X-100 (0.2%). Sialic acid then was quantified by the method of Warren (1959).

### Electron Microscopy

All tissue was examined on a JEOL 100 CX microscope. A carbon replica cross-grating (2160/µm) was photographed with each series of photographs for calibration.

### Freeze-Fracture Electron Microscopy

Samples of intact myocardium and pelleted isolated SL were rapidly frozen in Freon 22 at the liquid-solid interface. Frozen tissue was transferred to the cold stage of a Balzers 301 Freeze-Etch Unit equipped with an electron beam gun and a quartz-crystal monitor. Fracture was carried out at ~120°C in a vacuum of 3 × 10⁻⁷ torr. Fractured surfaces were immediately shadowed at an angle of 45° with 2 nm of platinum-carbon. The platinum replica was stabilized by deposition of pure carbon from a gun mounted at a 90° angle to the specimen plane. The replicas were floated sequentially through 20% household bleach in buffer to full-strength bleach. Washing was in doubly distilled water.

### Thin Section Electron Microscopy

Isolated SL vesicles were centrifuged to form a loose pellet, exposed to 2% glutaraldehyde or a combination of glutaraldehyde plus 2% tannic acid, and postfixed in 1% OsO₄. The tissue was dehydrated in ethanol and embedded in Epon. Sections (~60 nm) were cut with a diamond knife in a Porter-Blum MT2 ultramicrotome.

### Stereological Procedure

All replicas were photographed in the microscope at 30,240×. The negatives were projected and magnified 10x directly on a viewing screen for counting (final magnification, 300,000×). A 15-mm test circle was placed randomly on the F face of intact SL, TT membrane, and SR membrane. The number of IMPs enclosed by the circle was counted. The 15-mm test circle corresponded to an area of 1.88 × 10⁻⁶ µm². For isolated SL vesicles, the same 15-mm circle was placed on concave profiles free of cast shadow. This was reasonable, since we were able to determine that a considerable percentage of the vesicles were inside out (see section on vesicle sidedness). However, to ensure that counting only from this orientation
did not skew the data, we determined IMPs/\mu m^2 on some convex surfaces (77 vesicles in each dog and rabbit SL). All the IMPs totally enclosed within the circle were counted. Those IMPs that intersected the test circle were counted, alternately, in and out.

For intact dog myocardium, 320 circles (area = 0.6 \mu m^2) were counted on SL membranes (from 107 different cells), 102 circles (area = 0.2 \mu m^2) on TT membranes, and 74 circles (area = 0.14 \mu m^2) on SR. In SL isolated from dog, 527 vesicles were counted (one circle per vesicle, total area = 1.01 \mu m^2). For intact rabbit myocardium, 107 circles (area = 0.2 \mu m^2) were counted on SL membrane, 75 circles (area = 0.15 \mu m^2) on TT membranes, 74 circles (area = 0.14 \mu m^2) on SR membranes, and 320 circles (area = 0.6 \mu m^2) on isolated SL vesicles.

Vesicle Sidedness

Freeze-fracture morphology of isolated SL vesicles can be used to determine whether the vesicles enclose protoplasmic space and, thus, are oriented right side-out, or whether they enclose the extracellular space and are inside-out. The determination is based on the fact that cellular membranes have unequal densities of IMPs on their two fractured faces. The P face (closer to the cytoplasm) contains 6-8 times the number of IMPs as the E face (closer to the extracellular space) (Frank et al., 1980; Rayns et al., 1975). Isolated vesicles that are right side-out when fractured will have the higher IMP density on the convex surface and fewer IMPs in the concave surface. If, however, the membrane has vesiculated inside out, the concave surface will contain high IMP density and the convex surface will contain fewer IMPs.

Statistical Analysis

A general mixed-model analysis of variance program (BMDP3V) was used for testing differences between mean IMP densities from different membranes in a nested model in which hearts and replicas within hearts are random.
factors. The error variance is derived from the variability among areas in the same replica. The resulting pairwise comparison of means were used to compute the test for significant differences.

Results

Whole Muscle Fracture Faces

The characterization of membranes from freeze fracture preparations of intact tissue by means of IMP density was restricted to P faces of the peripheral SL, TT membrane, and SR membrane. A low-magnification freeze-fracture replica which contains areas from these various membranes is shown in Figure 1. In the overall view of the cell afforded by Figure 1, several TTs are seen. The TT is highly curved, and irregularly flanked by junctional SR. P faces of the TTs appear as troughlike concavities oriented perpendicular to the fiber axis, and contain many IMPs. The E face is convex, contains fewer IMPs, and clearly illustrates the tubular nature of the T system.

Fractures occurring longitudinal or transverse to the fiber axis produce areas of TT membrane sufficient for counting IMP densities (Fig. 2). Quantification of IMP densities on P faces of both TT membranes and the peripheral SL gave similar results (Table 1). In terms of IMPs/μm², there was no significant difference between TT membranes and SL for both dog and rabbit hearts. Mean values of the P face IMP densities are 2901/μm² for SL and 2807/μm² for TT in the dog. The rabbit myocardium yielded slightly (although not significantly) higher values, with SL = 3010/μm² and TT = 3005/μm². The value of IMP density in isolated rabbit SL is higher than 2208/μm², as determined in our previous study in intact myocardium (Frank et al., 1982). In that study, sarcolemmal IMP density was determined by counting IMPs in square micron areas of membrane (planimetry), as opposed to the stereological technique applied in this study. However, we cannot rule out the possibility that differences may relate to nonmyocytic membrane contamination in SL preparation.

Compared with SL and TT, the SR membranes have a much higher IMP density on their P face. The junctional and free SR in dog and rabbit myocardium consists of tubules with widths between 20 and 60 nm (Sommer and Johnson, 1979). Whereas

![Figure 2. Freeze-fracture electron micrograph where the fracture plane has exposed the P face of the transverse tubular membrane (TTp) and of the SR membrane (SRp). 78,300×.](image)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Membrane</th>
<th>Mean*</th>
<th>Mean ± 1 SEM (68% range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>Isolated SL (concave face)</td>
<td>2875 (527, 1.01)†</td>
<td>2828-2927</td>
</tr>
<tr>
<td></td>
<td>Intact SL</td>
<td>2901 (320, 0.60)</td>
<td>2838-2973</td>
</tr>
<tr>
<td></td>
<td>Intact TT</td>
<td>2807 (102, 0.20)</td>
<td>2713-2901</td>
</tr>
<tr>
<td></td>
<td>Intact SR</td>
<td>4938 (74, 0.14)</td>
<td>4730-5151</td>
</tr>
<tr>
<td></td>
<td>Isolated SL (convex face)</td>
<td>2729 (77, 0.15)</td>
<td>2701-2943</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Isolated SL (concave face)</td>
<td>3344 (320, 0.60)</td>
<td>3265-3427</td>
</tr>
<tr>
<td></td>
<td>Intact SL</td>
<td>3010 (107, 0.15)</td>
<td>2921-3103</td>
</tr>
<tr>
<td></td>
<td>Intact TT</td>
<td>3005 (75, 0.15)</td>
<td>2901-3109</td>
</tr>
<tr>
<td></td>
<td>Intact SR</td>
<td>4552 (75, 0.15)</td>
<td>4349-4771</td>
</tr>
</tbody>
</table>

* Analysis was in logs for stabilizing variances. Data obtained by taking anti-logs of values from analysis.
† Numbers in parentheses are, respectively, number of circles counted and area (μm²) of membrane quantified.
the fracture plane does not expose large areas of membrane, it frequently fractures enough free SR membrane flat enough for IMP counting (Fig. 3). The IMP densities for free SR membranes are given in Table 1. Mean values of the P face IMP densities are 4938/μm² in the dog and 4552/μm² in the rabbit. These values are significantly different from IMP/μm² values found in the SL and TT membranes (P < 0.001).

The most important observations in intact tissue are: (1) there is a significant difference in IMP density between SR and SL membranes and (2) that SL and TT have essentially identical IMP counts. A graphic distribution of the IMP density in intact hearts and isolated vesicles is given in Figure 4.

Purified Isolated SL

The isolated SL vesicles used in this study have been shown biochemically to be highly enriched in SL membrane (see Methods). The most distinctive marker for SR membranes is the ATP-dependent Ca pump, but, since SL also possesses this activity, it is difficult to estimate the SR contamination of an SL preparation. We have used the following approach. SL vesicles were loaded with Ca (~30 nmol Ca/mg protein) by the ATP-dependent Ca pump. EGTA and NaCl (70 mM final) then were added to activate Na⁺-dependent Ca efflux (Na-Ca exchange), an activity present in SL but not SR vesicles. After 1.0 minutes, 84% of the Ca was lost due to Na⁺-de-
Another new approach we used to assay for purity was to first load SL vesicles with Ca by passive diffusion. Vesicles were incubated at 4°C overnight in the presence of 1.5 mM labeled Ca. Aliquots containing about 35 nmol Ca/mg protein then were diluted into a KCl medium containing EGTA in the presence and absence of 70 mM NaCl. About 70% of the internal Ca could be released by external Na. This would imply that 30% of the vesicles in the SL preparation do not originate from the SL. However, this estimate is an upper limit, since it requires that all SL vesicles possess high Na-Ca exchange activity. Whether the Na-Ca exchange mechanism is homogeneously distributed in the SL membrane is unknown.

Figures 5 and 6 are thin section and freeze-fracture electron micrographs of a typical pellet from the SL preparation. Several important points emerge from studying these figures. From the thin section electron micrographs, the mean vesicle diameter was calculated to be 0.22 μm (Weibel, 1973). The vesicle membrane appears to lack portions of the glycocalyx. This was further investigated after enhancement of the cell surface with tannic acid fixation, as seen
in Figure 7. The bilayer shows an asymmetric density of stain. The outer leaflet is more intensely stained than the inner leaflet. The glycolcalyx (surface coat and external lamina) is not as dense as seen in intact cardiac tissue (Frank et al., 1982). It is most likely that the surface coat portion of the glycolcalyx is intact, and that some or all of the external lamina is no longer present. After tannic acid staining, the isolated vesicles were seen in closely associated clumps, such that portions of the vesicle membranes adhered to one another. This was a predominant finding after tannic acid staining, but was found infrequently in conventionally fixed pellets.

From the freeze-fracture morphology, it is clear that many concave as well as convex surfaces of the vesicles contain high IMP densities. Right side-out SL vesicles would be expected to have the greater IMP density on their convex surface (P face). Concave surfaces with high IMP density could occur either with inside-out SL vesicles or right side-out SR vesicles. Heavy contamination of the isolated SL with SR is not likely since (1) biochemical measurements did not indicate SR contamination (see above), and (2) the IMP counts clearly show a single peak with a mean value similar to that for intact SL, and do not show any significant shoulder in the curve where you would expect to see SR, namely, around 4000 IMPs/μm² (Table 1; Fig. 4). The average IMP density in isolated SL from the dog, determined from concave surfaces, is 2875/μm² and for rabbit 3344/μm². The IMP density was essentially superimposable on that found in intact SL and TT membranes (see Fig. 4). Thus, the morphological data support the homogeneity of these vesicles. Figure 8 shows the typical P and E faces seen in the isolated vesicles. IMP density on the P face of convex vesicles was not significantly different from IMPs counts found in P face of concave vesicles (Table 1). The SL membranes prepared (as described in Methods) from both dog and rabbit hearts were found by freeze-fracture morphology to have more vesicles inside-out than right side-out (Table 2).

The sidedness of the SL vesicles from canine cardiac muscle was also analyzed by biochemical techniques. Na,K-ATPase activity (determined by measurement in the presence and absence of KCl) was assayed under control conditions and in the presence of alamethicin (~1:1, alamethicin:protein; donated by Dr. J.E. Grady, The Upjohn Co.) or monensin (10 μM). Results are shown in Figure 9. Alamethicin unmask the latent enzyme activity in all sealed vesicles (Jones et al., 1980), whereas the ionophore, monensin, unmasks the activity of only inside-out SL vesicles by allowing K to reach its catalytic site at the extracellular SL surface within the inside-out vesicles. In the absence of monensin,
K would be rapidly depleted from the inside-out vesicles. Although these interpretations involve assumptions (discussed below), the data allow us to estimate that 45.3, 31.0, and 23.7% of the vesicles are right side-out, inside-out, and leaky, respectively. Similar results are obtained if valinomycin (3.6 μM) is used instead of monensin and if the detergent SDS is used instead of alamethicin.

To confirm our interpretation of the Na,K-ATPase data, experiments were performed, using the inhibitors ouabain and digitoxigenin (Fig. 10). Ouabain is membrane impermeable, whereas digitoxigenin readily permeates SL membranes. If control Na,K-ATPase activity (measured in the absence of monensin and alamethicin) is due to leaky SL vesicles, then both ouabain and digitoxigenin should inhibit enzyme activity. This was found to be the case. If monensin activates only the Na,K-ATPase activity of inside-out vesicles, then the monensin-stimulated component of Na,K-ATPase activity should be inhibited by digitoxigenin but not by ouabain. This is because the site of inhibitor action is on the internal surface of inside-out vesicles. This site is accessible to the permeable digitoxigenin but not to ouabain. Figure 10 shows that, indeed, ouabain does not block the component of Na,K-ATPase activated by monensin, consistent with our interpretation. After treatment with alamethicin, both ouabain and digitoxigenin give greater than 90% inhibition of enzyme activity (not shown). Similar analyses of Na,K-ATPase data have been reported recently (Seiler and Fleischer, 1982; Caroni and Carafoli, 1983).

Sialic acid measurements showed that the vesicle preparation has 73.4 ± 10.6 nmol sialic acid/mg protein and that 34.6 ± 3.4 nmol sialic acid/mg protein could be removed by neuraminidase. If the vesicles were first disrupted with detergent, then 57.8 ± 4.5 nmol sialic acid/mg protein could be removed by neuraminidase. Assuming that the increase in sialic acid susceptible to neuraminidase after vesicle disruption is due to inside-out vesicles, we calculate that 40.0 ± 3.8% of the vesicles are inside-out (n = 6). The remaining 60% of the vesicles would be right side-out vesicles, plus those vesicles leaky to the neuraminidase. It is commonly found, in membrane studies, that all sialic acid cannot be released by neuraminidase.

Discussion

Stereological analysis of freeze-fractured membranes from intact dog and rabbit myocardium was used as a reference with which to compare structural parameters of isolated SL membranes. The densities of IMPs observed on the fracture face (P face) of intact SR and surface membranes were very different (see Table 1). The intact free SR had considerably more IMPs (4938/μm² in dogs; 4552/μm² in rabbits) than the respective SL membranes (2801/μm² in dogs; 3010/μm² in rabbits). This agrees with similar measurements on skeletal muscle (Rayns et al., 1975; Franzini-Armstrong, 1975). Our measurements were confined to free SR, as this produced the largest expanse of membrane flat enough for counting. The IMP density was uniform throughout this portion of the SR and appeared to be a reflection of the distribution of Ca,Mg-ATPase in this part of the cardiac SR (Deamer and Baskin, 1969; Jorgensen et al., 1982).

The surface membrane displayed fracture faces similar to those of the T tubules. Table 1 gives the IMP density for the P faces of these membranes. In both dog and rabbit hearts, the IMP density distributions for P face of SL and TT are essentially superimposable (see Fig. 4). This is to be contrasted with skeletal muscle, where the IMP distribution in
the peripheral SL is quite different from that of the TT membrane (Frazinini-Armstrong, 1974; Scales and Sabbadini, 1979). In the several species of skeletal muscle examined, the P face of the TT membrane had fewer IMPs than the peripheral SL. In addition, thin section microscopy indicates TTs in skeletal muscle lack the external lamina coat present on surface of SL (McNutt and Fawcett, 1974). Recent biochemical studies of isolated mammalian skeletal TT membrane indicate a unique enzymatic, phospholipid, and protein composition of this membrane (Rosenblatt et al., 1981). Cardiac TT membranes have not yet been isolated. Thus, no similar biochemical data are available for the heart. Given the similar freeze-fracture profiles of TT and SL membranes, and the fact that the cardiac TT membranes contain both surface coat and external lamina, as present on the SL, one might not expect a different biochemical composition.

The transport data derived from isolated SL have been used to help understand Ca exchange in intact muscle. One major objective of this study was to compare the structure of isolated SL from dogs and rabbits to that of intact cells. It appears, from the thin section microscopy, that the cell surface of isolated SL is different from the intact myocardium. Tannic acid staining indicates less glycocalyx (defined here to include surface coat plus external lamina) than that found in intact myocardial cell membranes (Frank et al., 1982). Presumably, a portion of the external lamina is lost during the isolation procedure (see Figs. 5 and 7). It is not known how the lack of the external lamina might affect membrane function in isolated SL.

Figure 4 illustrates freeze-fracture IMP density distribution of isolated SL, along with the IMP distributions of intact SL, TT, and SR. The curves for intact SL, TT, and isolated SL membranes are nearly identical (Fig. 4). The pronounced asymmetry in IMP disposition (2807/µm² on the P face vs. 350/µm² on the E face) of intact SL is maintained in the isolated vesicles (Fig. 8). This, coupled with the fact that the vesicles have P face IMP counts similar to that of intact SL, demonstrates that loss of integral proteins from the membrane does not occur to any significant degree during the isolation procedure. However, some change in sidedness of isolated vesicles does occur. The fact that—in both dog and rabbit preparations—there were significant numbers of concave vesicles with IMP densities equivalent to those of the SL P face, indicates an inside-out vesiculation. Our estimates of inside-out vesicles, based on these morphological criteria are high—65% in the dog SL and 56% in the rabbit SL (Table 2). Whereas freeze-fracture morphology is an excellent method to assess sidedness, two factors should be considered: (1) Our estimates would include all vesicles, regardless of whether they were sealed or leaky. Functional criteria for sidedness (e.g., Na,K-ATPase measurements) can exclude leaky vesicles. (2) Contamination with other membranes could influence the results. SR contamination is likely to be low (see above), but would increase the estimate of inside-out SL vesicles. In addition, we cannot rule out the possibility of some contamination by outer mitochondrial membranes which have IMPs/µm² similar to SL (Packer, 1973). Two studies (Bers, 1979; Weglicki et al., 1980) have suggested the presence of outer mitochondrial membranes in isolated SL preparations. If the P face of the outer mitochondrial membrane was oriented in a concave direction, this might also increase our estimate of inside-out vesicles. In addition, the possibility exists that some vesicular contamination may arise from nonmyocytic cells (i.e., endothelial, smooth muscle, etc.).

The estimate of SL vesicle sidedness, from measurements of Na,K-ATPase activity, indicate that the vesicles are 45% right side-out, 31% inside-out, and 24% leaky. Thus, of the tightly sealed SL vesicles, about 60% are right side-out and 40% are inside-out. The validity of these estimates relies on various assumptions. The method requires that sealed vesicles have no Na,K-ATPase activity. The Na,K-ATPase needs substrates (Mg, ATP, Na, K) on both intra- and extracellular membrane surfaces, and we assume that substrate diffusion must be too slow to affect the data if particle-free vesicles were assumed to be E faces.

Table 2

<table>
<thead>
<tr>
<th>Method</th>
<th>% RSO ± SEM</th>
<th>% ISO ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1400, 20, 150</td>
<td>34.5 ± 5.1</td>
<td>65.5 ± 5.6</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000, 20, 150</td>
<td>43.8 ± 4.4</td>
<td>56.2 ± 4.6</td>
</tr>
</tbody>
</table>

RSO = right side-out vesicles; ISO = inside-out vesicles. Method 1 included only vesicles that had particles. Method 2 counted all vesicles. Particle-free vesicles were counted as E face. These two methods for determining sidedness were necessary, since we could not be sure that particle-free vesicles always represented an E face orientation. It is possible, although unlikely, that a particle-free surface represented uncleaved vesicles. Measurements performed by both approaches showed that it did not affect the data if particle-free vesicles were assumed to be E faces.

* Number in parentheses are in the order given: total vesicles counted, number of replicas examined, and number of electron micrographs.
maintain significant enzyme activity; it is unlikely that this assumption introduces major errors. It is also assumed that neither alamethicin nor monensin has any direct effect on Na,K-ATPase activity. Their effects are interpreted solely in terms of increased substrate accessibility. Since the effects of alamethicin and monensin may be mimicked by detergent and valinomycin, respectively, this assumption is probably valid. The sialic acid measurements indicate that 40% of the vesicles are inside-out. This result depends on the assumption that all cellular sialic acid is located on the extracellular surface of the SL. This has not been verified in cardiac muscle. The calculation also requires that the neuraminidase does not have access to the interior of any vesicles. In a recent study, we estimated that 33–48% of the SL vesicles are inside-out, based on measurements of Na-Ca exchange (Philipson and Nishimoto, 1982), but we have since been unable to consistently reproduce the experiments, using polymixin and digitonin from which this estimate was made. In an earlier study (Bers et al., 1980), we estimated that only 20% of the SL vesicles in a preparation from rabbit heart were inside-out. We have since modified the procedures for SL preparation and sidedness determination and have obtained higher estimates of inside-out vesicles. In addition, we have now used freeze fracture morphology as a powerful tool for sidedness estimation.

Considering the approximations inherent in both morphological and biochemical approaches, the qualitative agreement of the estimations of vesicle orientation is noteworthy. All techniques indicate that there is a substantial fraction of both inside-out and right side-out vesicles present, and that the bilayer in both types of vesicles is similar in its morphology to intact membrane.

We wish to thank G. Mottino for his expert technical assistance throughout this study. This research was supported by Grants HL 28791 and HL 27821 from the U.S. Public Health Service, National Heart, Lung, and Blood Institute, J.S. Frank is the recipient of a Research Career Development Award 1 K04 HL 01072 from the National Institutes of Health. K.D. is the recipient of an Established Investigatorship from the American Heart Association.

Address for reprints: J.S. Frank, Ph.D., Cardiovascular Research Lab, A3-381 CHS, UCLA School of Medicine, Los Angeles, California 90024.
Received December 1, 1983; accepted for publication February 1, 1984.

References
Bers DM (1979) Isolation and characterization of cardiac sarclemma. Biochim Biophys Acta 533: 131–146

INDEX TERMS: Isolated sarc lemma • Freeze fracture • Membrane sidedness
Ultrastructure of isolated sarcolemma from dog and rabbit myocardium. Comparison to intact tissue.

J S Frank, K D Philipson and S Beydler

Circ Res. 1984;54:414-423
doi: 10.1161/01.RES.54.4.414

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1984 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/54/4/414