Structure of the Freeze-Fractured Sarcolemma in the Normal and Anoxic Rabbit Myocardium

JOY S. FRANK, SARAH BEYDLER, MICHAEL KREMAN, AND ERIC E. RAU

SUMMARY The purpose of this study was to examine the ultrastructure of the sarcolemma in the normal and severely anoxic rabbit heart with the technique of freeze-fracture. Severe anoxia and subsequent reoxygenation cause a significant decrease (31%) in intramembranous particles (IMP) in the P face of the membrane and a 25% decrease in the E face. P face IMP's are severely aggregated. The decrease in density and the redistribution of IMP's indicate a severely altered lipoprotein structure of the sarcolemma. In addition, the necks of caveolae open and the caveolae become flattened in the plane of the membrane. With reoxygenation, many rupture. Spherical projections of cytoplasmic vesicles appear in the membrane (possibly of sarcoplasmic reticulum or lysosomal origin) and also can be seen to rupture after reoxygenation. When glucose is present in the perfusate, it affords some protection against these structural defects. We propose that the fragmentation or holes in the sarcolemma reported in severe anoxia are directly related to the structural changes reported in this study.


THE technique of freeze-fracture has been used increasingly in electron microscopy to study the morphology of the cell membrane (Devine and Rayns, 1975; Bullivant, 1974; McNutt, 1975). Replicas of freeze-fractured membranes contain images of intramembranous particles or more complex structures such as caveolae dispersed in the plane of the membrane with various densities and degrees of order. Cell membranes from a variety of cells have been described qualitatively with the technique, and a few studies have been quantitative (Ellisman, et al., 1976; Weinstein, 1968; Kirk and Tosteson, 1973; Branton, 1969). However, an extensive analysis of this sort has not been made for the myocardial cell membrane. Several qualitative descriptions of cardiac muscle using the freeze-fracture technique have been provided (McNutt, 1975; Rayns et al., 1968; McNutt and Weinstein, 1970; Ashraf and Halverson, 1977), but none has quantified intramembranous particles or as yet described quantitative membrane changes with experimental interventions. The goal of this study was 2-fold: (1) to quantify the density of intramembranous particles and caveolae of the myocardial cell membrane under well-defined control conditions and (2) to compare these structures in the membrane under conditions of severe anoxia and subsequent reoxygenation. The initial part of this study serves as a reference point by which physiologically important changes in membrane structure can be quantified and related to membrane function. The anoxic myocardium was contrasted with the control myocardium because of previously reported changes in its membrane permeability (Burton et al., 1977; Rau et al., 1977), in its ultrastructure as revealed by routine electron microscopy (Hatt and Moravec, 1971), and its biochemistry (Scheuer, 1967; Lai and Scheuer, 1975). Ultrastructural studies on prolonged anoxia show a disruption of the sarcolemma which is manifest by gaps or holes in the unit membrane (Ganote et al., 1975; Hearse et al., 1975; Feuvray and Deliris, 1975). However, the sequence of structural changes that leads to this fragmentation of the membrane and thus to irreversible cell injury is not known. Standard thin-section electron microscopy affords little structural detail on cell membrane changes. This may be because many important membrane specializations lie in the plane of the membrane and cannot be discerned in thin sections. The present freeze-fracture study describes several changes in the structure of the sarcolemma which are directly related to the membrane fragmentation and massive enzyme release characteristic of reoxygenation after prolonged anoxia.

Methods

Preparation of the Muscles

The isolated, interventricular septum of a 1.5 to 2.0-kg male New Zealand white rabbit was perfused through its arterial supply according to techniques previously described (Rau et al., 1977). The septal artery was cannulated and perfused at a constant rate (1.8 ml/g per min) by a Harvard pump at 28°C and stimulated at 42 beats/min. Developed tension was measured throughout the experiment. The standard perfusate contained (mm): NaCl, 130; NaHCO₃, 12; MgCl₂, 1.0; NaH₂PO₄, 0.435; dextrose,
5.5; and CaCl₂ 1.5. After equilibration of the solution with 98% O₂-2% CO₂, the pH was 7.3-7.4.

Septa were allowed to equilibrate for 60 minutes with perfusate after cannulation. In control muscles, the perfusate was switched to 2% glutaraldehyde in 0.2 M Na cacodylate buffer without interruption of the flow to the tissue. Perfusion with the fixative was continued for 20-30 minutes, the muscle cut down, minced into small pieces (<1 mm on a side), and placed in glutaraldehyde for 1 hour. After fixation, the muscle was either treated for routine microscopy as previously described (Frank and Langer, 1974) or processed for freeze-fracture electron microscopy (see below).

In addition to the perfused septal preparation, control experiments were done on a rabbit papillary muscle immersed in a bathing solution that was renewed at a perfusion rate of 2 ml/min. This was done to ensure that our quantification of intramembranous particles was independent of the preparation used and could indeed be a basis for comparing structural changes in the membrane. A heart from the rabbit was rinsed in oxygenated perfusion medium (given below) and then transferred to a dissection dish which was gassed constantly around its perimeter with 98% O₂ and 2% CO₂. A papillary muscle, 0.35 mm in diameter, was dissected out and kept at constant length while being transferred to the perfusion chamber. Contractile force was measured throughout the experiment. The muscle was fixed in glutaraldehyde while still tied to the chamber.

Anoxia

Anoxia was induced by switching to identical perfusate which was equilibrated with 98% N₂-2% CO₂ (pH 7.3-7.4). The O₂ content of the N₂-CO₂ equilibrated solution was undetectable by Lex-O₂ Con (Lexington Instruments) determinations.

All experiments on anoxia used the interventricular septal preparation. The following types of experiments were done: (1) 2 hours of anoxia without substrate; (2) 2 hours of anoxia without substrate but with subsequent reoxygenation (10 minutes); (3) 1 hour of anoxia without substrate; (4) 2 hours of anoxia with substrate (5.5 mM glucose); and (5) 2 hours of anoxia with substrate plus reoxygenation. Reoxygenation was accomplished by returning to solution equilibrated with O₂ and CO₂.

Freeze-Fracture Electron Microscopy

After glutaraldehyde fixation, pieces of muscle were placed in 25% buffered glycerol for 1 hour. Whereas some tissue was rapidly quenched in liquid freon 22 cooled to its freezing point by liquid N₂, the bulk of the tissue was frozen using the equipment described by Sjostrand and Kretzer (1975). Gold alloy hats (3-mm diameter, Balzer High Vacuum) containing tissue were frozen in liquid propane at -180°C, liquid N₂ being used to cool the propane. The temperature of the propane was kept uniform by rapid stirring. These precautions were taken to prevent the formation of ice crystals during freezing. The fracturing and shadowing were done in a Balzers Freeze-Etch Unit BAF 301. The transfer of the specimens to the precooled specimen table (-170°C) was accomplished with a specially designed stainless steel cover that fit on the specimen carrier and provided a large cold mass over the tissue at all times during the transfer. Fracturing was performed at -150°C at a vacuum of 5 x 10⁻⁷ torr or better. Shadowing (45° angle) was done with a Balzers Electron Beam Unit (EVM 052) set to deposit 20 Å of platinum-carbon. The replicas were cleaned in bleach and distilled water before mounting on 300-mesh grids. All electron microscopy observations were made on a Siemens 1A microscope. For calibration, a photograph of a carbon replica cross-grating (2160 lines/mm) was taken with each series of photographs.

Quantification of Intramembranous Particle Density

Particle densities per μm² of membrane were determined by projecting and magnifying (final magnification 300,000×) the negative directly on a viewing screen with a Nikon profile projector (6C). An acetate sheet was placed on the viewing screen and, as each particle was counted, it was marked in ink on the acetate sheet overlay. For each 0.25 μm² of membrane counted, the acetate sheet with the generated density map was xeroxed for a permanent record and the sheet cleaned and re-used. In all instances, care was taken to avoid areas where excessive curvature of the membrane exaggerated the shadowing of the particle. The particles counted were 85-100 Å in diameter. On the E face, particle size is quite variable. Only particles that were 85-100 Å were counted.

Caveolar necks in the membrane were counted in the same manner, only from low magnification negatives (5000×) where areas of membrane 1 μm² or greater were fractured.

Results

Structure of the Myocardial Sarcolemma: Intramembranous Particles

All freeze-fractured myocardial cells were characterized by two distinct fracture faces, the P face (adjacent to the cytoplasm) and E face (adjacent to the extracellular space). The intramembranous particle densities for P and E fracture faces are given in Table 1. Fracture face P had 2300 ± 43 (SEM) intramembranous particles per μm² of cell membrane. This mean value is from cells of the interventricular septum and from cells of the isolated papillary muscle. There was no significant difference between the two preparations, indicating that as long as muscle was prepared carefully it made no...
The presence of glucose in the perfusate during the anoxic stress afforded some protection to the membrane since there was no noticeable aggregation of particles on the P face (Fig. 6). In addition, the loss of particles was considerably less with glucose present—16% and 5% for the P and E faces, respectively (Table 1).

### Caveolae and Spherical Projections

The other structures of the sarcolemma clearly revealed by freeze-fracture preparations were the openings of the t tubules (Fig. 4) and the small invaginations (40–70 μm) similar to the micropinocytotic vesicles called caveolae (Fig. 1). The caveolae were fractured at their necks with the necks appearing as rising structures on the E face and as depressions on the P face. We found 4.7 caveolar necks per μm² of membrane (48 μm² counted; Table 2).

Changes in the structure of caveolar necks and frequency per μm² became evident after 2 hours of anoxia without substrate. The caveolar necks were less frequent, decreasing by almost 50% to only two necks per μm² (Table 2). The caveolae also appeared flattened in the plane of the membrane and were shaped as domes (Fig. 5, bottom). The exposed membrane of the “dome” or cavity of the caveolae contained very few intramembranous particles. Reoxygenation after 2 hours of anoxia had a pronounced effect on the caveolae. The necks on both faces were essentially absent with less than one caveolar neck per μm² in over 103 μm² of membrane (36 cells) counted (Fig. 4). The caveolae still present were completely open or flattened in the plane of the membrane. They were recognizable by their “dome” configuration and almost particle-free membrane (Fig. 5, bottom). The sarcolemma appeared flat and free of membrane folds, but there were frequent holes or tears in the membrane (Figs. 4, bottom, and 5, bottom).

In addition, after 2 hours of anoxia, there appeared in the plane of the membrane projections on the P face and a corresponding arrangement of depressions on the E face (Fig. 4, top and bottom). These projections were scattered throughout the membrane, were approximately 40–80 nm in diameter, and quite often several projections or “bumps” formed a “rosette” configuration (Figs. 3, right and 4, top). After 2 hours of anoxia, these projections appeared to be fusing (Fig. 3, left and right) or, with subsequent reoxygenation, forming an opening in the membrane (Fig. 5, top right). The orientation of these projections was just the opposite of that seen with caveolae and indicated that they were probably vesicles in the cytoplasm deforming the cell membrane. In thin sections, there were numerous large vesicles projecting into the cell membrane (Fig. 7, bottom left) which appeared to correspond to the projections seen in the plane of the freeze-fractured membrane.

When glucose was in the perfusate during the anoxic period, the spherical projections were not present, and the caveolar necks had essentially the

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**Table 1** Membrane Particle Densities for Control and Anoxic Myocardium

<table>
<thead>
<tr>
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<th>Fracture face P (particles/μm²)</th>
<th>Fracture face E (particles/μm²)</th>
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<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2300 ± 43 (19)</td>
<td>332 ± 29 (10)</td>
</tr>
<tr>
<td><strong>Anoxia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 Min NS</td>
<td>2117 ± 77 (10)</td>
<td>326 ± 43 (10)</td>
</tr>
<tr>
<td>120 Min NS</td>
<td>1767 ± 50 (11)</td>
<td>250 ± 23 (10)</td>
</tr>
<tr>
<td>120 Min + ReO₂ NS</td>
<td>1579 ± 70 (10)</td>
<td>226 ± 29 (10)</td>
</tr>
</tbody>
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Each entry is mean number particles per μm² ± SEM. Numbers in parentheses represent the number of μm² analyzed for each fracture face. NS = no substrate (i.e., dextrose) in the perfusion medium during anoxic stress; S = substrate present throughout anoxic period; ReO₂ = reoxygenation for 10 minutes after anoxia.

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The presence of glucose in the perfusate during the anoxic stress afforded some protection to the membrane since there was no noticeable aggregation of particles on the P face (Fig. 6). In addition, the loss of particles was considerably less with glucose present—16% and 5% for the P and E faces, respectively (Table 1).
FIGURE 1 Freeze-fracture replica of normal myocardial cell sarcolemma. Top: P fracture face (inner membrane half) contains numerous intramembrane particles, 62,200 x. Bottom: E fracture face (outer membrane half) has few particles. Caveolar necks (depressions on P face, rising stumps on the E face) are numerous (arrows), 66,960 x. Bar = 0.2 µm.
**Figure 2** Left: Higher magnification replica of P face of normal sarcolemma showing the density and distribution of 85–100 Å particles. 116,500 ×. Right: Same as left but for the E face. 117,180 ×. Bar = 0.1 μm.

**Figure 3** Portion of the sarcolemma after 2 hours of perfusion without oxygen and substrate. Left: Replica shows spherical projections (arrows) on P face. 119,070 ×. Right: Depressions (arrows) in rosette pattern in the E face. 115,900 ×. Bar = 0.1 μm.
FIGURE 4  Sarcolemma from the myocardium after 2 hours of anoxic perfusion without substrate and with subsequent reoxygenation. Top: the P face. 55,080 x. Bottom: the E face. 55,080 x. Bar = 0.2 μm. Notice the absence of caveolar necks on both faces, the aggregation of particles on the P face, the spherical bumps on the P face (arrows), and the depressions on the E face (arrow). T = transverse tubule.
FIGURE 5  Higher magnification of freeze-fractured sarcolemma illustrating the important changes in membrane structure after 2 hours of anoxia with subsequent reoxygenation. Top left: shows severe clustering of particles on P face and decrease in density of particles. 85,680 ×. Top right: Spherical bumps projecting from cytoplasm; a projection (arrow) appears to be bursting. 109,620 ×. Bottom: Area of sarcolemma (P face) after 2 hours of anoxia illustrating caveolae flattened in the plane of the membrane (arrows). Note particle-free appearance of caveolar membrane. In the left hand side of the micrograph, the membrane has torn (arrowhead). 113,750 ×. Bar = 0.1 μm.
same structure and density as in control hearts (Table 2, Fig. 6, left and right).

**Thin-Section Microscopy**

Thin-section microscopy on the anoxic myocardium and on the anoxic myocardium with subsequent reoxygenation showed similar ultrastructural changes to those reported previously (Hearse et al., 1975; Hatt and Moravec, 1971). After 1 hour of anoxia without substrate, there was some slight intracellular edema, slight mitochondrial swelling, and essentially normal sarcomeres with no myofibrillar disruption (Fig. 7, top left). After 2 hours without oxygen and substrate, the cells did show marked intracellular swelling, mitochondrial swelling, and severely contracted sarcomeres (Fig. 7, bottom left). With subsequent reoxygenation, the intracellular edema became severe, the mitochondria were very swollen with disruption of the cristae, and the sarcomeres were no longer intact. The sarcolemma had extensive peeling of the glycocalyx, and the unit membrane then appeared fragmented with actual gaps in its structure (Fig. 7, bottom right). Thin sections of the glucose-protected myocardium showed a lack of intracellular swelling, normal mitochondria, and normal sarcomeres (Fig. 7, top right). There were no tears or gaps in the unit membrane, but the separated glycocalyx was still present (Fig. 7, top right).

**Capillary Endothelial Cells**

Although we did not do as intensive a study on endothelial cells as on the myocardial cells, it was clear that anoxic stress caused severe structural changes in the capillaries. We looked only at fracture planes passing through membrane on the tissue front for this comparison and specifically areas not over the nucleus. We found the necks of the pinocytotic vessels to be ~40 nm in diameter with a density of 51/μm² (range 45–70). Even after 1 hour of anoxia, there was a decrease in the number of pinocytotic vesicles to 21/μm², whereas after 2 hours of anoxia and subsequent reoxygenation,

**TABLE 2**

<table>
<thead>
<tr>
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<th>Caveolar necks/μm²</th>
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<tr>
<td>Control</td>
<td>4.7</td>
<td>(48)</td>
</tr>
<tr>
<td>Anoxia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 Min NS</td>
<td>4.5</td>
<td>(34)</td>
</tr>
<tr>
<td>120 Min NS</td>
<td>2.0</td>
<td>(34)</td>
</tr>
<tr>
<td>120 Min + ReO₂ NS</td>
<td>0.8</td>
<td>(103)</td>
</tr>
<tr>
<td>120 Min + ReO₂ S</td>
<td>4.0</td>
<td>(72)</td>
</tr>
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</table>

Numbers in parentheses represent the number of μm² analyzed. NS = no substrate in the perfusion medium; S = substrate present; ReO₂ = reoxygenation for 10 minutes.
FIGURE 7 Thin-section electron micrographs from the anoxic myocardium. Top left: 1 hour of anoxia without substrate. Note peeling of glycocalyx (arrow) and slight cellular edema but otherwise normal structures. Mit = mitochondria; Mfi = myofibrils. 44,100 x. Bar = 0.5 μm. Top right: 2 hours of anoxia with subsequent reoxygenation but with substrate present. Slight cellular edema and peeling of the glycocalyx is present (arrow). The Mfi are not contracted, and Mit look normal. 42,000. Bar = 0.5 μm. Bottom left: 2 hours of anoxia without substrate. Note large swollen vesicles under the sarcolemma (asterisk) and severely contracted sarcomeres. 35,000 x. Bar = 0.5 μm. Bottom right: 2 hours of anoxia with subsequent reoxygenation without substrate. There is extensive peeling of the glycocalyx (arrows), gaps in the unit membrane, disruption of Mfi and mitochondrial Mit and cytoplasmic swelling. 31,800 x. Bar = 0.5 μm.
there was severe aggregation of intramembranous particles in the P face (Fig. 8, right).

**Discussion**

In the present study, we quantified the intramembranous particles on both fracture faces and the caveolae in the normal and in the anoxic sarcolemma, a perturbation known to affect membrane function (Nayler et al., 1979). The vascularity perfused interventricular septum of the rabbit was the preparation used since its ionic exchange properties are well characterized under a variety of circumstances including anoxia and ischemia (Rau et al., 1977; Nayler et al., 1979; Shine et al., 1978).

**Control**

As in most tissues examined, so far, the P face of the membrane had the higher density of particles. We found $2300 \pm 43$ particles per $\mu m^2$ on the P face and $332 \pm 29$ particles per $\mu m^2$ on the E face. Quantitative studies on intramembranous particles on the fracture faces of other cells have reported similar particle counts. The red blood cell membrane has been studied most extensively in this regard with very good agreement among laboratories for the P face (average values 2600/$\mu m^2$) but with a large variability in E face counts (Weinstein, 1968; Kirk and Tosteson, 1973; Branton, 1969). The E face contains a more heterogeneous population of particles than does the P face. All particles are not spherical, and some appear almost as rods. The complexity of the E face and its functional significance deserve further study.

In 1968, Rayns et al. in a study on freeze-fracture of guinea pig heart sarcolemma found 400-700 particles per $\mu m^2$ on the P face and 80-120 particles per $\mu m^2$ in the E face. These exceptionally low figures (lower than reported for any membrane analyzed so far) differ significantly from the data of this study. Perhaps the best explanation is the very different protocols used for handling the tissues. These investigators injected 30% glycerol directly into the beating myocardium without previous fixation. Treatment of unfixed tissue with glycerol recently has been found to decrease the number of intramembranous particles (Kirk and Tosteson, 1973; McIntyre et al., 1974), whereas fixation with glutaraldehyde has been found to prevent glycerol-induced changes (McIntyre et al., 1973). However, although there has been good correlation between the appearance of freeze-fracture replicas from some glutaraldehyde-fixed tissue with unfixed tissue frozen in physiologic saline, chemical fixation of
tissue also can cause structural alterations (Raviola et al., 1978).

Caveolae have been characterized in rat and rabbit hearts (Levin and Page, 1977; Gabella, 1978), in skeletal muscle (Dulhunty and Franzini-Armstrong, 1975), and in smooth muscle (Gabella, 1976), but their function remains obscure. In the freeze-fracture sarcolemma, the caveolae usually are fractured at the level of their necks, with 2–3 cavities per neck. The caveolar membrane shows strikingly few particles in the P face. In the rat, the density of caveolar necks is 6.1/μm² (Gabella, 1978), whereas in rabbit ventricle, a density of 3.7 necks per μm² has been reported (Levin and Page, 1977). The caveolae in our present study had a very similar density of 4.7 caveolar necks per μm². The differences between the rabbit and rat probably represent species differences, since the rat has well known functional (Langer, 1978) and, perhaps, structural anomalies.

Anoxic Stress

Several studies support the hypothesis that severe alterations of membrane function are of key importance in the evolution of irreversible injury in the anoxic heart (Nayler et al., 1979). Although changes in membrane permeability to cations seem to occur very early in anoxic stress (Rau et al., 1977; Burton et al., 1977), they appear to be reversible, since recovery after reoxygenation is very good until the heart has been anoxic for an hour or more (Burton et al., 1977). Studies using lanthanum (La³⁺) as a probe for changes in membrane permeability clearly demonstrate that the entrance of La³⁺ into the cell after 1 hour of anoxia is reversible, but by 2 hours, La³⁺ deposition within the cell is irreversible (Burton et al., 1977). When reoxygenation occurs after approximately 100 minutes of anoxia, there is an immediate 100- to 200-fold increase in the release of cellular enzymes (Hearse and Humphrey, 1975). In thin sections, the cells have marked intracellular edema, mitochondrial edema, and severely contracted or disrupted sarcosomes. The earliest structural change in the sarcolemma seen with thin-section microscopy is the separation of the glyocalyx from the unit membrane (Ganote et al., 1975; Hearse et al., 1975; Feuvray and DeLeiris, 1975). This is a consistent ultrastructural defect (see Fig. 7) as yet poorly understood and similar to the glyocalyx peeling described under conditions of zero-calcium perfusion (Frank et al., 1977; Crevey et al., 1978; Muir, 1967). After prolonged anoxia (approximately 100 minutes or more), gaps or holes have been noted in the unit membrane (Ganote et al., 1975; Hearse et al., 1975) which become very noticeable in tissue examined after reoxygenation. The sequence of structural changes in the membrane leading to this fragmentation has not been described. Presumably they are related to the reduction of cellular energy supplies, cellular swelling, the marked mechanical stress of contracture, and the abnormal cellular permeability to Ca²⁺ which have been described in severe anoxia (Nayler et al., 1979; Ganote et al., 1977; Ganote and Kaltenbach, 1979).

In our present study, we found a decrease in the density and marked aggregation of the intramembranous proteins, a decrease in the number of caveolar necks per unit area of membrane, and we noted spherical projections from the cytoplasm deforming the membrane. All of these changes were severe after 2 hours of anoxia without substrate but were markedly exacerbated after reoxygenation (see Figs. 4 and 5; Table 1).

Clearly, the significant (P < 0.005) reduction in the number of intramembranous proteins, as well as a reorganization in their distribution in the lateral plane of the membrane, characterized by aggregation, is related to altered lipid and protein interactions. The altered lipoproteins in the membrane may be directly responsible for the changes in permeability of the cell occurring in hearts deprived of oxygen. A growing number of observations on other tissues have related altered membrane permeabilities directly to aggregation and loss of intramembranous particles (Kirk and Tosteson, 1973; Bourguet et al., 1976; Coleman and Duggan, 1976). We can only speculate at this time as to the cause(s) of the observed clustering and decrease in number of particles per unit area of membrane that occur with prolonged anoxia. However, it is tempting to relate relevant data from other tissues to our present findings. In ischemic injury of liver cells, Chien et al. (1978) have shown that membrane-bound phospholipases are activated as a result of increased cellular Ca²⁺ shown to accompany ischemia. Freeze-fracture of isolated membranes showed a decrease in number and aggregation of intramembranous proteins. Although there are no data as to whether endogenous phospholipases are released in the myocardium, this is an intriguing possibility. The increased cytoplasmic free Ca²⁺ concentration after 2 hours of anoxia could activate membrane phospholipase (Nayler et al., 1979). Degradation of phospholipids by exogenous phospholipase C has been shown to alter cell permeability and cause severe aggregation of intramembranous particles in myocardial cells grown in culture (G. A. Langer, J. S. Frank, and K. Philipson, unpublished observation). Most likely, no one mechanism will be responsible for structural alterations in membrane lipoproteins. Reduction in ATP levels in late anoxia may have a direct effect on membrane structure as well. In rat red blood cells, depleted of ATP, the freeze-fractured membrane showed clustering of intramembranous particles (Gazitt et al., 1976). Depletion of ATP, which causes a decrease in the phosphorylation of membrane proteins, might alter the interaction between protein and lipids.

In addition to the altered lipoprotein interac-
tions, the sarcolemma is subjected to the mechanical force of marked intracellular edema. Reoxygenation after 2 hours of anoxia causes augmentation of this swelling and release of intracellular enzymes. Insight into the mechanism of membrane tears may be found in the present data on the caveolae. The changes in the density of caveolae per unit area from 4.7 caveolar necks per μm² in controls, to 2.0 caveolae per μm² in anoxia of 2 hours duration, to less than 1 caveola per μm² after 2 hours of anoxia with subsequent reoxygenation can be related to the amount of intracellular edema present during the anoxic stress.

With increased intracellular swelling, the caveolae are flattened in the plane of the membrane but are recognized by their particle-free appearance (Fig. 5C). The flattening of caveolae in the plane of the membrane, along with a decrease in membrane folds, probably accounts for the greater than 50% reduction in caveolae per μm² of membrane reported here. In extreme swelling of the cell, as occurs with reoxygenation, all the caveolae open, and this probably accounts for our data of less than one neck per μm². With increased swelling, the membrane ruptures producing the tears seen in freeze-fracture and the gaps in the unit membrane seen in thin sections (Hearse et al., 1975; Ganote et al., 1975, 1977). The ability of the caveolae to open is presumably a complex function of their surface tension and of the already altered lipoprotein of the membrane. Similar openings of the caveolae necks have been reported in skeletal muscle under conditions of extreme stretch to the fibers and under conditions of cellular swelling induced by hypotonic media (Dulhunty and Franzini-Armstrong, 1974).

In addition, cellular swelling causes spherical projections protruding into the membrane from the cytoplasm. They are seen as bumps in the P fracture plane and depressions in the E face (Fig. 4). In thin sections, these projections are viewed as subsarcolemmal blebs or vesicles (Fig. 7C). They could be of lysosomal origin or originate from swollen vesicles of the sarcoplasmic reticulum. In the plane of the fractured membrane, they are frequently grouped in a “rosette” pattern and, with reoxygenation, they appear to rupture (Fig. 5B).

The membrane is thus subjected to the mechanical stress of: (1) the opening of the caveolar necks into the plane of the membrane and to (2) the spherical vesicles deforming the membrane from the cytoplasm. The mechanical forces are acting on a membrane with severe lipoprotein alterations and, as a result, may cause the fragmentation of the membrane characteristic of irreversible anoxic stress and cell death. Hearse et al. (1975) have reported on the protective effect of glucose in the perfusion medium. They find a reduction in enzyme loss and improved preservation of myocardial ultrastructure. Our data support this protective effect since glucose appears to protect the membrane from lipoprotein alterations. The intramembranous particle density decreased by 16% when glucose was in the medium as opposed to a decrease of 31% in substrate-free perfusate (see Table 1). Aggregation of the particles was very slight, there was considerably less intracellular edema, and the caveolae had an essentially normal density and configuration (Fig. 6; Table 2).

A final consideration arises from our present data and concerns the capillary cell membrane in the anoxic heart. The endothelial cell membrane of the capillary undergoes structural changes similar to those seen in the sarcolemma. The intramembranous particles are severely aggregated, even more so than the sarcolemma after a similar interval without oxygen, whereas the micropinocytotic vesicles clearly show changes. There is a significant decrease (50%) in the density of the vesicles per μm² of membrane, with many vesicles flattened in the plane of the membrane. Whereas damage to capillary endothelial cells has been an important consideration in ischemia (Kloner et al., 1974), little attention has been given to the oxygen-deprived endothelial cells in anoxia. It is not known how soon after oxygen loss the structural changes we noted become apparent. The severity of the changes at 2 hours strongly suggests a compromised exchange across the capillary, and the effect of this on the anoxic myocardial cell warrants consideration.

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Erratum
In the Brief Review by M.R. Malinow, entitled “Atherosclerosis: Regression in Nonhuman Primates” (Circ Res 46: 311-320, 1980), Figures 2 and 4 were printed incorrectly and should have been in reverse order.
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