Ultrarapid Freezing Reveals That Skeletal Muscle Caveolae Are Semipermanent Structures

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Rat myofibers in the resting state and under imposed conditions of moderate to severe physiological, pharmacological, and mechanical stress were prepared by ultrarapid freezing and examined by freeze fracture. In rapidly frozen rat myofibers, caveolae morphology and distribution were found to be unchanged by brief or prolonged rest, brief direct electrical stimulation and concomitant contractile activity, prolonged direct electrical stimulation (to fatigue), myofiber stretch (within normal myofiber limits), or careful compressive scission. However, caveolae were greatly reduced or eliminated in number and size by severe mechanical disruption (shredding and/or tearing) of myofibers. Thus, we conclude that unlike apparently similar surface specialization in other cell types, skeletal muscle caveolae are not transient stages in a caveolae-vesicle endocytotic-exocytotic cycle, nor are they a membrane reservoir for normal stretch/contractile activity. Rather, they are (semi)permanent structures in the muscle plasma membrane with as yet undetermined function and kinetics.  © 1986 Academic Press, Inc.

In thin-section images of muscle plasma membranes, caveolae are observed as Ω-shaped (omega-shaped) indentations or as shallow depressions of the plasma membrane (Bundgaard, 1983; Franzini-Armstrong et al., 1975). In freeze-fracture replicas of aldehyde-fixed muscle, caveolae are seen either as cup-like or saucer-like P-face indentations having distinctive borders, or as truncated conical E-face projections of the plasma membrane (Bonilla et al., 1981; Dulhunty and Franzini-Armstrong, 1975; Shotton, 1982). Several models have been proposed concerning the role of caveolae in a variety of tissues (Bonilla et al., 1981; Bundgaard, 1983; Dulhunty and Franzini-Armstrong, 1975; Franzini-Armstrong et al., 1975; McGuire and Twietmeyer, 1983; Prescott and Brightmann, 1976; Steinman et al., 1983). Their function in skeletal muscle has been suggested to be a reservoir of membrane that becomes incorporated into the sarcolemma during myofiber stretch (Dulhunty and Franzini-Armstrong, 1975). In other tissues, however, caveolae-like structures have been proposed as reversible sites for endocytosis or exocytosis (Steinman et al., 1983) or as equivalent to the vesicle fusion sites for water and/or ion exchange (Linder and Staehelin, 1979), but there is no direct evidence to favor any of these concepts for muscle caveolae.

Although the function of caveolae is not yet established, several recent freeze-fracture studies of caveolae in glutaraldehyde-fixed skeletal muscle have focused on caveolae alterations as a possible means of characterizing neuromuscular diseases (Bonilla et al., 1981; Costello and Shafiq, 1979; Lee et al., 1986; Shotton, 1982). Contradictory data in these reports concerning perceived changes in the number, size, and distribution of caveolae in normal and diseased muscle led members of this group to examine the relationship between method of chemical fixation and differences in caveolae preservation (Lee et al., 1986). We reported that caveolae are predictably altered based on the mode of glutaraldehyde ex-

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posure (immersion vs perfusion). Moreover, as glutaraldehyde fixation techniques were progressively improved, caveolar diameter (as the degree to which caveolae were distributed in bands) also progressively decreased according to the following sequence: immersion-fixed central myofibers > immersion-fixed surface myofibers > perfusion-fixed myofibers. Thus, we concluded (Lee et al., 1985) that the more rapidly a myofiber is exposed to a high concentration of fixative, the smaller the diameter and the more irregularly distributed the caveolae.

Since the primary variables of that study (and of all previous studies of muscle caveolae) were time, diffusion distance, and method of aldehyde exposure, the observed variability in caveolae preservation was attributed to temporal, spatial, or chemical artifacts of aldehyde fixation. Moreover, those data seemed to support suggestions that fixation-dependent changes in caveolae size reflected inadequate or delayed preservation of an extremely transient physiological process (i.e., that caveolae represent transient states in the sequence: free cortical vesicles ↔ attached vesicles ↔ caveolae). According to that model, different modes of chemical fixation preserve the caveolae at different states of fusion and/or detachment from the plasma membrane. Consequently, ultrarapid freezing was considered as a method to study caveolae dynamics because this method of ultrastructural preservation provides the necessary time resolution and alternative to chemical fixation to contribute to a resolution of this controversy.

Recent studies of caveolae in rapidly frozen endothelia and fibroblasts (Bretscher and Whytockey, 1977; McGuire and Twietmeyer, 1983) indicate that there is a significant decrease in the number of caveolae as compared with the number in chemically fixed cells of the same type. In addition, a significant decrease in the diameter of caveolae was reported in rapidly frozen aortic endothelial cells as compared with that in chemically fixed cells (McGuire and Twietmeyer, 1983). Likewise, an initial examination of rapidly frozen rat skeletal muscle sarcolemma by members of this group (Lee et al., 1983) gave preliminary data indicating that rapid freezing drastically reduced the number and size of caveolae in muscle plasma membrane. Consequently, this detailed investigation was conducted to determine whether caveolae were, in fact, extremely transient structures. Improved techniques (Boyne, 1979) that allow rapid freezing of surface fibers in intact whole muscles rather than in muscle fragments (Lee et al., 1983) allowed us to examine the presumed sequence and time course of caveolae formation and turnover under a wide variety of physiological and nonphysiological conditions. We systematically investigate the effects of instantaneous tissue preservation on caveolae morphology and distribution and investigate the presumed sequence and time course of the proposed cyclic changes in caveolae morphology. We rapidly freeze intact and cut myofibers under a wide variety of conditions (rest, normal electrical and contractile activity, extreme conditions of altered muscle physiology and pharmacology, and extreme mechanical stress) to determine which factors (if any) maximize, minimize, or alter the presumed high rate of caveolae turnover. Thus, we presumed that this systematic examination would allow us to determine whether the caveolae variabilities noted by us and by others were related to normal muscle activity, to artifacts of specimen preparation, or to other factors.

MATERIALS AND METHODS

Sarcolemmal caveolae of rat extensor digitorum longus (EDL) muscles were prepared by rapid freezing under a wide variety of conditions: (1) following brief rest (normal control), (2) following prolonged (drug-enforced) rest, (3) immediately following brief stimulation, (4) following prolonged stimulation (to fatigue), (5) during moderate muscle stretch, (6) following myofiber scission and stretch, and (7) following lateral shear of the myofibers. For each condition, three whole EDL muscles were surgically removed (one each from three normal male Wistar rats, 29–44 days old, 112–170 g). Prior to excision of the EDL muscles from one group
of rats, animals were anesthetized by intraperitoneal injection with sodium pentobarbital (64 mg/kg) [a centrally acting "sedative hypnopic" with general depressant activity, including action on peripheral nerves and muscles (Price, 1975)]. To ensure that possible changes in caveolae diameter were not due solely to the method of anesthesia used, a second group of rats was anesthetized with ketamine (87 mg/kg)-xylazine (13 mg/kg), both of which are nonnarcotic centrally acting muscle relaxants with little or no effect on peripheral nerve or muscle activity (Price, 1975; Haver-Lockhart, 1983). A heat lamp was used during surgery to maintain the normal body temperature of the rats. To maintain tissue homeostasis, surgically exposed or excised muscles were kept moist with 37°C oxygenated rat Ringers buffer (pH 7.2). All excised tissues were mounted on Polaron aluminum rapid freeze disks and immediately frozen to liquid nitrogen temperature using the Boyne (1979) "Gentleman Jim" rapid freeze device. In all cases (except for the experiments to test the effects of cell scission; see below), less than 1 min expired between excision and rapid freezing of the tissue.

(1) Brief Rest (Control)

Unstimulated EDL muscles were exposed, excised, and rapidly frozen, as described above. Since nerve scission during tissue removal produces a brief burst of nerve depolarizations and since the resulting stimulation might result in caveolae alteration and an inaccurate determination of sarcolemmal caveolae in resting myofibers, additional muscles were prepared under conditions in which nerve scission could not induce muscle contraction (i.e., during pancuronium-induced neuromuscular blockade; see below).

(2) Prolonged/Enforced Rest (Neuromuscular Blocking Agent)

To ensure that the muscles sampled had been in the resting state (i.e., not neuronally stimulated) for at least 30 min before rapid freezing, pancuronium [a "stabilizing" neuromuscular blocking agent (Koelle, 1975)] was administered 30 min prior to the excision of EDL muscles. After each rat was anesthetized, a tracheotomy was performed for mechanical respiration during neuromuscular blockade. The peroneal nerve to the left EDL muscle was exposed and stimulus pulses (0.5 msec pulse duration, 2 V) were delivered at 10 Hz in 2-msec trains via a bipolar stimulating electrode, resulting in a normal twitch response. The femoral vein was exposed and pancuronium (2 mg/kg) was administered by intravenous injection. At the onset of respiratory blockade, each rat was respirated. Indirect neural stimulation of the left EDL resulted in no twitch response, but direct electrical stimulation of the same muscle elicited a normal twitch response, thereby demonstrating a complete and maintained whole-body neuromuscular blockade. This test stimulation regimen was repeated every 10 min during the 30-min enforced rest period to ensure continued maintenance of complete neuromuscular blockade. After 30 min, the unstimulated contralateral (right) EDL muscle was carefully exposed, excised, and rapidly frozen, as above.

(3) Brief Stimulation

Intact exposed EDL muscles were directly stimulated via bipolar stimulating electrode using a supramaximal stimulation consisting of four 6-V pulses. 2 msec in duration. The muscles then were excised and rapidly frozen (15–30 sec after stimulation), as above.

(4) Prolonged Stimulation (to Fatigue)

To ascertain whether prolonged stimulation resulted in accumulation of or disappearance of caveolae, intact exposed EDL muscles were fatigued by direct moderate frequency stimulation (20-Hz, 6-V repeated pulses, 2 msec in duration) until there were no visible signs of muscle twitch (1–2 min to muscle fatigue, total of 1000 to 2000 contractions). Muscles then were excised and rapidly frozen, as above.

(5) Maintained Stretch

Whole EDL muscles were removed, stretched to about 150% of rest length, and immobilized at that length on aluminum rapid freeze disks. The ends of each muscle were secured in slots at the end of each disk (Fig. 1) to maintain muscle stretch during the rapid freezing step, which followed immediately. Samples were rapidly frozen, as above.

(6) Cut Fibers

(a) Five-millimeter segments. Whole EDL muscles were removed and immersed in 37°C oxygenated rat Ringers buffer (pH 7.2). The muscles were immediately halved longitudinally, quartered horizontally using a sharp razor blade, and left in rat Ringers buffer for 5 min. The pieces of quartered muscle then were rapidly frozen, as above.

(b) One-millimeter segments (compression-cut fibers). To ensure that the only mechanical damage in-
CAVEOLAE IN ULTRARAPIDLY FROZEN MUSCLE

TABLE I

<table>
<thead>
<tr>
<th>Condition</th>
<th>No./100 μm²</th>
<th>No./μm²</th>
<th>Mean diameter (nm) ± SD</th>
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</thead>
<tbody>
<tr>
<td>(1) Brief rest−control</td>
<td>1190</td>
<td>11.9</td>
<td>62.7 ± 22</td>
</tr>
<tr>
<td>(2) Prolonged/enforced rest</td>
<td>1057</td>
<td>10.6</td>
<td>70.7 ± 25</td>
</tr>
<tr>
<td>(3) Brief stimulation</td>
<td>1051</td>
<td>10.5</td>
<td>58.1 ± 16</td>
</tr>
<tr>
<td>(4) Prolonged stimulation</td>
<td>1271</td>
<td>12.7</td>
<td>64.8 ± 24</td>
</tr>
<tr>
<td>(5) Maintained stretch</td>
<td>1177</td>
<td>11.8</td>
<td>61.4 ± 26</td>
</tr>
<tr>
<td>(6) Cut fibers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 5-mm segments</td>
<td>1101</td>
<td>11.0</td>
<td>63.4 ± 25</td>
</tr>
<tr>
<td>(b) 1-mm segments (compression cut)</td>
<td>1057</td>
<td>10.6</td>
<td>65.9 ± 28</td>
</tr>
</tbody>
</table>

Note. At least one 25-μm² area well frozen myofiber from each rat is included in the 100 μm² analyzed.

Freeze Fracture and Electron Microscopy

Rat muscle fibers were fractured and replicated in a Balzers 301 freeze-etch device (Balzers, Hudson, NH) and prepared for electron microscopy according to our published methods (Hudson et al., 1981; Rash et al., 1981). To maintain the integrity of each replica during the required cleaning stages, the frozen replicated samples were coated with Lexan according to the method of Steere and Erbe (1983). After cleaning and mounting of replicas, the Lexan was removed by rinsing the grids in three changes of ethylene dichloride. Replicas were examined in a Philips 400T transmission electron microscope (Philips Electronic Instruments, Mahwah, NJ) operated at 80 kV. The freeze-etch nomenclature of Branton et al. (1975) is used in this report.

Quantitative Analysis

Caveolae were counted and their diameters measured on ×10,000 micrographs using a ×10 Bausch & Lomb scale lupe. A total area of 100 μm² of well-frozen sarcolemma (25 μm² from each of four ×10,000 micrographs, one micrograph from each of four different myofibers) was analyzed for each muscle condition. No more than two fibers from the same animal were analyzed. Well-frozen sarcolemma was identified by smooth, ripple-free membrane surfaces (indicating the absence of large ice crystals) and by the presence of distinct, "nonstringy" intramembranous particles [indicating the lack of membrane compression (Ornberg and Reese, 1979)]. We note, however, that in addition to caveolae, a few of the openings in the sarcolemma represent openings of T-tubules (Dulhunty and Franzini-Armstrong, 1975; Franzini-Armstrong et al., 1975) but it is not possible to differentiate between these two distinct structures using current freeze-fracture techniques. However, previous freeze-fracture investigations examining caveolae morphology and number reported their data as the sum of these two structures. To facilitate comparison, we use the same convention.

RESULTS

Freeze-fracture replicas of plasma membranes (sarcolemmas) of rapidly frozen EDL muscles are shown in Figs. 2–4. Caveolae morphology and distribution were compared in sarcolemmas of muscle fibers obtained from rats anesthetized with the two different anesthetics and treated according to each of the six protocols. Within the four
25-μm² membrane areas analyzed for each condition, the number of caveolae ranged from 10 to 13/μm² while the mean caveolar diameters ranged from 52 to 72 nm. The pooled data (100 μm² of total pooled membrane area) for each of the experimental conditions (except for the ripped and torn fibers) revealed that there were 10 to 13 caveolae/μm² and that their mean diameters ranged from 58 to 73 nm (see Table 1).
These values were consistent within each of the four samples from each condition, as well as between all fibers from all experimental conditions (except for torn fibers). Caveolae were found to be distributed randomly under almost all conditions. (Representative micrographs from only three of the six conditions are shown because the results were nearly identical for all conditions, with the exception of ripped and/or torn myofibers; see below.) On rare occasions small areas of sarcolemma were observed where caveolae were present in bands. However, the appearance of banding was neither definite, distinctive, nor consistent. This is in contrast to that seen in immersion-fixed myofibers (Lee et al., 1986; Bonilla et al., 1979; Shotton, 1982).

Charts 1–6 graphically illustrate the number of caveolae present at each diameter in the 100 μm² of sarcolemma analyzed for each condition. Similar caveolae size ranges of 10–190 nm were observed in each group. Note that the curves in each graph are similar, indicating that caveolar diameter and number of caveolae present at each diameter are consistent under all normal physiological conditions and under several non-physiological conditions. A comparison of mean diameters (X) and standard deviations (SD) in Chart 7 graphically reveals that there were no statistically significant differences in these parameters following any of the pretreatment conditions (except tearing).
The only exceptions to these data suggesting the relative stability of skeletal muscle caveolae were observed in the 1-mm segments obtained from laterally sheared fibers and in fibers stripped or torn from intact muscles. In both instances, large expanses of well-frozen sarcolemma often were observed exhibiting none or very few caveolae, mostly as minute (10–20 nm) dimples (Fig. 5, arrows) but a few as conventional 70- to 100-nm caveolae. It is not known whether the small number of dimples in these damaged areas represented openings to T-tubules or the initial or final stages in the cycle of vesicle attachment → fusion → detachment, nor could we ascertain the fate of the caveolae that had disappeared. Even in stereoscopic images, no identifiable patches of membrane were discerned which could be attributed to caveolae that had become incorporated into the sarcolemma. In other areas of the same fibers, however, the numbers of caveolae were similar to those observed in intact myofibers from the other pretreatment conditions (i.e., 10 to 12/μm²). However, because of the extreme variability in the number of caveolae in fibers that had been torn or sheared, the data from these unusual conditions are not included in Table 1.

DISCUSSION

Caveolae in cells other than skeletal muscle have been reported to be extremely transient structures, often essentially absent (or present in much lower numbers) in rapidly frozen cells as compared with those in chemically fixed cells (Bretscher and Wytack, 1977; McGuire and Twietmeyer, 1983). In those systems, the observed large number of caveolae in chemically fixed cells is thought to result either from glutaraldehyde-induced fusion of subplasmalemmal vesicles with the plasma membrane, an artifically increased rate of normal vesicle fusion, or temporal summation during slow fixation of a normally high rate of vesicle fusion. Although glutaraldehyde is thought to increase the rate and probability of membrane fusion events (Chandler and Heuser, 1979), caveolae may nonetheless represent only one of the normal stages in a reversible fusion-detachment cycle. According to this widely accepted structural model, caveolae represent a reversible mechanism for endocytosis and/or exocytosis (Steinman et al., 1983).

This investigation utilized rapid freezing in an attempt to characterize the presumed time course of caveolae fusion and/or detachment from the plasma membrane in an electrically excitable and contractile tissue. Muscle cells in the resting condition (untrated) and under a variety of physiological and pharmacological conditions that were hoped to maximize and/or minimize caveolae formation/turnover were prepared by ultrarapid freezing. In contrast to our stated expectations (Lee et al., 1986), our data indicate that (a) in skeletal muscle, caveolae are semipermanent structures in the plasma membrane, (b) caveolae morphology and distribution are essentially unchanged by a wide range of normal physiological states (i.e., rest, stimulation, fatigue, stretch) or by myofiber scission, and (c) caveolae are altered (so far) only by severe mechanical disruption (lateral shearing or ripping and/or tearing).

Our data concerning the number, size, and distribution of caveolae in rapidly frozen muscle are explained most easily by the hypothesis that caveolae are semipermanent or long-lived structures with a very slow rate of turnover. If caveolae were short-lived structures with a rapid turnover related to normal excitation or contraction, the temporal summation and structural movements associated with continuous contractile activity during prolonged (multisecond) chemical fixation might be presumed to result in the capturing and stabilizing of many transient events occurring over several seconds as cortical vesicles fused with (or detached from) the plasma membrane. Indeed, if caveolae were transient morphological structures, they might be expected to
vary according to the state of excitation, contraction, or degree of muscle stretching. According to that model, elimination of temporal summation by the use of the instantaneous preservation technique of rapid freezing would, therefore, be expected to result in fewer sarcolemmal caveolae. Moreover, the ratio of the different morphologies would then be presumed to reflect the relative duration of each stage of the fusion-detachment cycle. A lower ratio of the number of caveolae found in rapidly frozen myofibers to the number found in chemically fixed myofibers would indicate that caveolae were short-lived structures. However, our findings from rapidly frozen muscle under a variety of conditions demonstrate that this ratio is near unity (i.e., the number of caveolae in rapidly frozen myofibers is virtually identical to the number in chemically fixed myofibers). Thus, these data indicate that caveolae are actually relatively long-lived structures with slow (or no) turnover rates. Under all tested conditions (except in torn myofibers, and at the ends of fibers damaged by lateral shear), we found caveolae in a variety of sizes ranging from 10 to 190 nm (X = 60 to 70 nm), suggesting that these long-lived structures either (a) cycle very slowly (i.e., many tens of seconds) or (b) are semipermanent structures that are normally present in variable sizes. Therefore, future studies of caveolae function should address other mechanisms of caveolae formation and turnover (i.e., nutritional state, hydration, myofiber growth, and aging).

These same findings indicate that the distinctive and reproducible variabilities in caveolae morphology and distribution reported in sarcolemmas fixed by several different chemical fixation protocols result from artifacts induced by an as yet unquantified aspect(s) of fixation chemistry. One important variable was shown to be the mode of exposure to the chemical fixative. Significant variability in caveolae was reported (Lee et al., 1983) based on how rapidly a myofiber was exposed to the fixative (immersion vs perfusion). In central fibers of immersion-fixed muscle, the caveolae were of much greater diameter than those in rapidly frozen samples. On the other hand, the consistently small diameter caveolae seen in perfusion-fixed muscle (which we presumed to be the most rapid means of exposing myofibers to chemical fixatives) were not observed in rapidly frozen muscle. Rather, the diameters in caveolae in rapidly frozen muscle were significantly larger than those in perfusion-fixed myofibers, and most nearly resembled those in surface fibers of immersion-fixed muscle. (This may mean that surface fibers are exposed to high concentrations of glutaraldehyde more quickly than are the fibers in perfusion-fixed muscle, where diffusion from the restricted volume of the capillary bed may delay fixation for several additional seconds and expose the fiber to slowly increasing concentration of fixative.) There also appear to be other aspects of fixation chemistry influencing these reported changes (i.e., hypoxia, acid production, and/or loss of positive charges on membranes (Johnson, 1985, 1986). Moreover, the current and previous data (Lee et al., 1986) raise additional concerns (Chandler and Heuser, 1979) as to the extent different chemical fixation methods induce artifactual changes in the morphology of other (membranous and cytoplasmic) structures.

Our findings also provide evidence that contradict and, at the same time, explain our preliminary observations that rapid freezing of rapidly dissected myofibers significantly reduced the size and number of sarcolemmal caveolae (Lee et al., 1983). The results of the mechanical trauma experiments indicate that the following factors led to those initial observations. In that preliminary study, minute muscle fragments were quickly dissected out (ripped and/or torn from whole muscles) and placed into the small freezing cavities of the propane jet device and rapidly frozen within 30 sec. For comparison, equally small pieces were quickly dissected out and rapidly frozen with
the Polaron “Slammer” freezing device. By duplicating those disruptive tissue sampling methods in one portion of this study, as well as by examining 1-mm muscle segments with laterally sheared ends, we determined that the methods used in the original sampling procedures resulted in the damaging of some surface fibers, thereby creating patches of sarcolemma that contained fewer and smaller caveolae. Thus, it appears that only the sarcolemmas of the most severely damaged myofibers were observed in that earlier study. Similar artifactual alterations of caveolae are considered likely using qualitatively similar “punch biopsy” and “needle biopsy” methods.

In another of our previous reports (Lee et al., 1986), we suggested rapid freezing as an alternative method of caveolae preservation in an attempt to characterize human neuromuscular diseases. Consequently, it became necessary to determine the morphology of caveolae in the natural (i.e., rapidly frozen) state and to identify preparative conditions that might alter caveolae morphology. Our findings in this investigation of rapidly frozen skeletal muscle indicate that several common physiological states (rest, stimulation, fatigue, etc.) have very little effect on caveolae morphology. Moreover, since we showed previously that glutaraldehyde fixation induces artifactual alterations in caveolae size and distribution but does not affect caveolae number, and since caveolae are now shown to be essentially unchanged by a variety of other pre-fixation conditions, the current data provide further support for the conclusion that the number of caveolae is consistently and characteristically increased in Duchenne muscular dystrophy (Bonilla et al., 1981; Shotton, 1982). Nevertheless, because of the substantial and unpredictable alterations of caveolae morphology that occur during relatively slower chemical fixation (Lee et al., 1986), the use of rapid freezing techniques may be required to determine whether the observed changes in caveolae morphology and distribution represent reliable pathologic alterations for characterizing neuromuscular disease.

Finally, the findings of this study increase our concerns as to whether chemical fixation, in fact, preserves the morphologic “reality” of cellular structures or causes misinterpretable artifact. Thus, it becomes increasingly important to determine the factor(s) of chemical fixation that may be inducing alterations in the morphology of these and other cellular structures.

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