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MYOCYTE SWELLING AND PLASMALEMMAL INTEGRITY DURING EARLY EXPERIMENTAL MYOCARDIAL ISCHEMIA IN VIVO

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Abstract

Using scanning and transmission electron microscopy, the structure of myocytes early in the phase of irreversible injury induced by 40 minutes of severe regional ischemia has been investigated, paying particular attention to the effects of cell swelling on the SEM appearance of the myocytes. Swollen myocytes showed an increased space beneath the plasmalemma and between organelles. True subsarcolemmal blebs were not seen and the attachment complexes between the Z-band and the underlying myofibrils remained intact. The proportion of the PS face of the plasmalemma which appeared "en face" (0.70%, SD:1.22 vs 5.01%, SD:3.72) in freeze-fracture faces of ischemic tissue was increased significantly. The increase may be due to swelling of the cell in the subplasmalemmal space or may be mediated through structurally unapparent alterations in the cytoskeleton. The PS face showed frequent longitudinal shifts in the alignment of Z band - plasmalemmal attachment between adjacent myofibers. T-tubule ostia, caveolae and junctional SR were demonstrable on the PS face. With standard preparative methods and instrumentation, no discrete fibrillar network associated with Z band attachment or subjacent to the plasmalemma was detected and there was no direct structural evidence of cytoskeletal alteration as a result of ischemic injury. Osmium-ligand preparation of tissue samples resulted in less damage induced by the electron beam, but did not allow significantly better resolution.

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Key Words: Myocardial infarction, sarcolemma, cell injury, cytoskeleton, freeze-fracture, osmium-ligand preparation, critical point drying.

Introduction

Metabolism occurring while myocytes are ischemic results in the production of large numbers of intracellular osmotically active particles. Intracellular water rises as a consequence of the osmotic imbalance; cell swelling is the structural manifestation of this phenomenon (Friedrich et al. 1981, Tranum-Jensen et al., 1981). Cell swelling is tolerated by myocytes during the phase of reversible injury, but when they become irreversibly injured, breaks in the sarcolemma appear. This change, unlike cell swelling, is incontrovertible evidence of irreversible cell injury and occurs sufficiently early to be the change which may cause the transition from reversible to irreversible injury (reviewed by Jennings et al., 1986).

Results of recent studies of the effect of anoxia on sarcolemmal integrity, suggest that sarcolemmal injury involves at least two steps, which appear to be independent of each other (Steenbergen et al., 1985). First, there is an ultrastructurally unapparent weakening of the plasma membrane or the cytoskeleton supporting it. This change does not result in overt rupture and appears to occur only after high energy phosphate supplies are depleted markedly. Secondary stresses, such as cell swelling, then lead to membrane rupture.

The influence cell swelling exerts on the structure of myocytes, the sarcolemma, and the attachments between the cytoplasmic face of the sarcolemma and subjacent myofibrils, has been investigated in this study, using freeze-fracture of fixed dog myocardium, and SEM. We compared myocardium known to contain a high proportion of irreversibly injured myocytes to non-ischemic control tissue. Particular attention was directed at the inner or true cytoplasmic face of the sarcolemma, because this is the site where cytoskeletal alterations related to plasmalemmal weakening might occur.

Materials and Methods

Tissue sampling

Ischemic and non-ischemic tissues were obtained from open-chest anesthetized dogs using techniques described in detail elsewhere (Jennings et. al., 1978). Ischemia was induced by ligation of the proximal left circumflex coronary artery for 40 minutes. Thioflavine S was injected intravenously 15 seconds before excision of the heart to identify the zone of severe ischemia and the heart was excised rapidly and was cooled in ice-cold KCl. The severely ischemic tissue in the posterior papillary muscle (PP) was free of TS when the heart was examined under UV light. This tissue was used for study. Brightly fluorescent well perfused anterior

papillary muscle (AP) was used as a source of control non-ischemic muscle. Tissue samples were cut into 2x2x4 mm (maximum dimension) blocks orientated parallel to the myocardial fiber direction in the muscle, and immersed in 4% glutaraldehyde in 0.1M cacodylate - sucrose buffer (pH 7.4, 318mOsm) for 24 h at 4°C, then rinsed and stored in the same buffer at 4°C.

SEM preparation

Conventional. Fixed tissue blocks were dehydrated in an ascending series of ethanol solutions ending in 3 x 10 min rinses in 100% ethanol. Some blocks then were sliced with a single-sided blade, which had been cleaned with solvent, using the technique of Sommer and Waugh (1976). Other blocks were frozen in thawing Freon-13, transferred to liquid nitrogen and fractured parallel to the long axis of the myofibers with a precooled single sided blade using the technique described by Ashraf and Sybers (1975), Ashraf (1982). Frozen fragments were thawed in 100% ethanol and, together with the fractured samples, were critical point dried using CO_2 as transition fluid in a Ladd critical point drier. Dried fragments were oriented and mounted on aluminum stubs with conductive silver paint, and coated with gold-palladium in a Polaron E5100 sputter coater (25kV, 50mm working distance, 180 sec).

Osmium-ligand. Similarly fractured or sliced blocks were prepared by the O-GTA-O-GTA-O (Takahashi - cited Murphy, 1980), GTA-O-TCH-O (Murakami and Jones, 1980), and OTOTO (Ashraf, 1982) techniques using the schedules and reagent concentrations described by the respective authors. Specimens were examined uncoated and following brief gold-palladium sputter-coating (40 sec).

TEM sample preparation

Fixed tissue blocks were dehydrated in ethanol and sliced into blocks <1mm on edge while in 100% ethanol, then rehydrated in buffer and post-fixed in 1% collidine buffered OsO_4 for 1 h, stained with uranyl acetate en bloc, embedded in Epon, ultrathin sectioned, and stained with lead citrate.

Microscopy

SEM samples were examined in secondary electron image mode in a JEOL JSM35C SEM at 15mm working distance, 15-35kV accelerating voltage, and images were photographed on Polaroid 55PN. Following convention (Branton et al., 1975) this face is termed the PS face to distinguish it from fracture faces created within the lipid phase of the membrane itself (PF and EF faces) and the true external surface of the cell (ES face). Also by convention (Sommer and Jennings, 1986) "sarcolemma" is used to refer to the membrane (plasmalemma) and surface lamina components combined.Thin sections were examined in JEOL 100B or Philips 410 TEM at 60-80kV.

Quantitation

The prevalence of "en face" portions of PS face of plasmalemma in fracture faces was determined by counting points falling on that plane from a rectangular array of 836 equidistant points at 1 cm spacing overlying the SEM viewing screen. Twenty non-overlapping fields (1000x magnification) forming a transect across the fracture face were counted for each of 6 samples of ischemic and of control papillary muscle. Lower and right margin points were excluded. The significance of the difference in the mean values was tested both after pooling the 6 samples (n = 120 x 2; Student's t-test), and without pooling (n = 20 x 12; analysis of variance), using an acceptance level of $p < 0.01$.

Results

General Appearance

Fracture faces of glutaraldehyde fixed control myocardium were composed predominantly of cell interiors and the ES (extracellular) membrane face. Cell interiors typically showed a planar face in which there was little space between the myofibrils (Fig. 1a). Nuclei were compact and showed finely granular chromatin. Space between the plasmalemma and underlying myofibrils was not seen in the plane of fracture usually elicited, i.e. parallel to the longitudinal axis of the cell. The intercellular space was small and the ES (extracellular) face of the plasmalemma almost always was obscured completely by the complex array of connective tissue which inserted into the dense fibrillar

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Figure 1a: SEM of a fracture plane passing longitudinally through the interior of three control cardiac muscle cells. The intercellular space separating them (arrow heads) is very narrow. The compact fracture face shows prominent Z-bands on myofibers (small arrows), dispersed nuclear chromatin (n) and small rounded mitochondria (m). There is little appreciable intracellular space. Bar = 10 µm.

Figure 1b: SEM of the external surface (ES face) of control cardiac muscle cells showing the large collagenous strands and bundles of the extracellular matrix inserting into the fibrillar surface lamina. The Z-fold periodicity (arrow heads) is evident through the lamina. Bar = 1 µm.

Figure 1c: Representative TEM of control cardiac muscle. Note the dispersed nuclear chromatin (n), and lack of appreciable intracellular spaces. Z-bands are not universally in register when adjacent myofibers are compared (arrow head). Mitochondria (arrow) are compact. Bar = 5 µm.

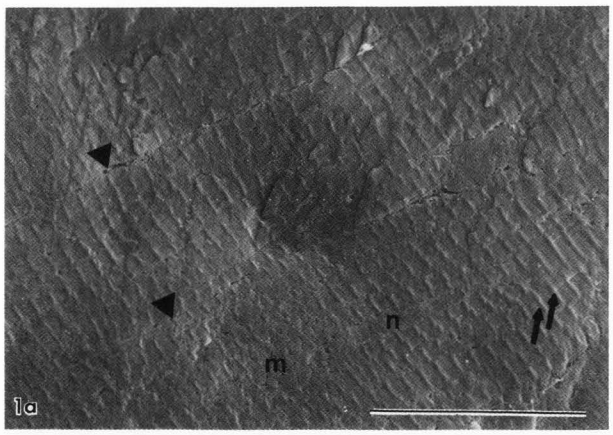
Figure 1d: Representative TEM of the periphery of a control dog cardiac muscle cell. The myofibrillar Z-band (Z) is closely apposed to the plasmalemma (arrow heads). The subplasmalemmal space is filled with organelles including junctional sarcoplasmic reticulum (fine arrow) and glycogen (broad arrow). Surface lamina and collagen bundles lie outside the plasmalemma. Bar = 0.5 µm.

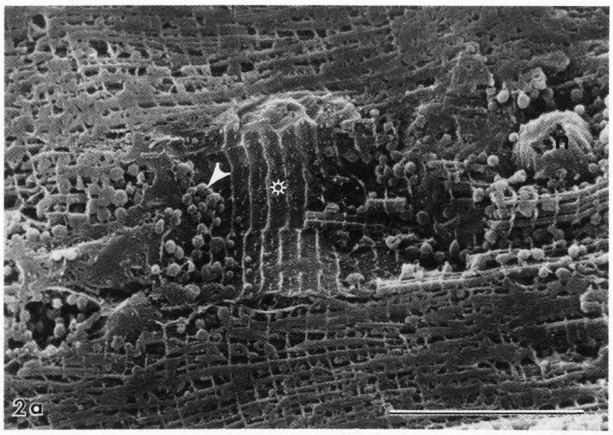
Figure 2a: SEM of cardiac muscle after 40 min of ischemia in vivo showing part of 3 cells. There is marked separation of myofibers and mitochondria by lucent spaces. The central cell shows an exposed patch of plasmalemmal PS face (asterisk) with adherent mitochondria (arrow head) and myofibrils, including a swollen nucleus containing clumped chromatin (N). Bar = 10 µm.

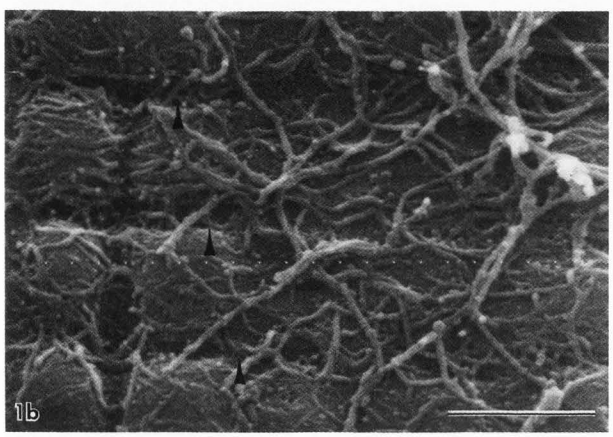
Figure 2b: SEM detail of PS-face seen in Fig. 2a. Z-folds appear as prominent ridges on the PS face (Z) and there are focally attached mitochondria and myofibrils. A longitudinal shift in Z-fold alignment is seen at center field. Bar = 1 µm.

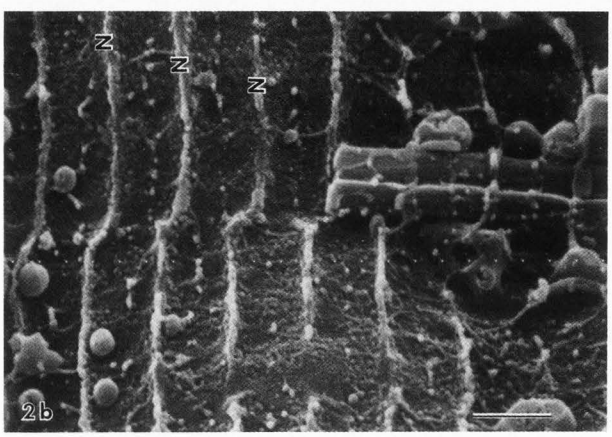
Figure 2c: Representative TEM of cardiac muscle ischemic for 40 min in vivo showing chromatin margination in the swollen nucleus (N), swollen mitochondria containing amorphous matrical densities (arrow) and extensive lucent spaces separating organelles. Myofibers are relaxed and show wide I-bands (arrow head), and there are areas of longitudinal shift in Z-band alignment between adjacent myofibers (asterisk). Bar = 5 µm.

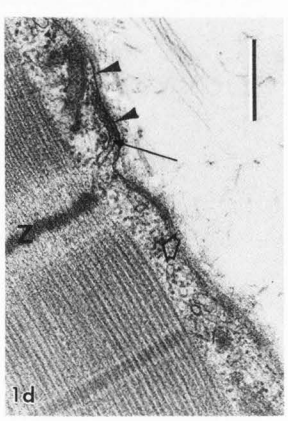
Figure 2d: Representative TEM of the periphery of an ischemic muscle cell. The plasmalemma remains attached at Z-band level (Z), and the subplasmalemmal space is expanded and relatively lucent (asterisk). Mitochondria are swollen (m) and contain matrical densities (arrow). Bar = 0.5 µm.

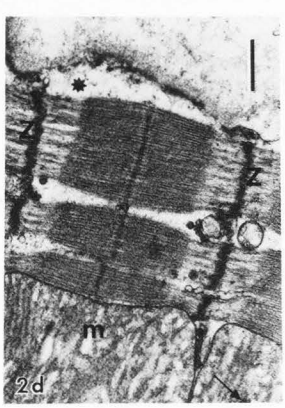












surface lamina (Fig. 1b). Despite this surface layer, sarcomere periodicity usually was obvious. Representative TEM appearances of control myocardium are shown in Figs. 1 c,d.

Fracture faces of ischemic myocardium showed changes consistent with cell swelling (Fig. 2 a-d). There were extensive small spaces separating the myofibrils, a change which allowed clearer delineation of mitochondria, which also were irregularly swollen. Despite the swelling, intermyofibrillar connections at Z-bands still were frequently apparent; these structures represent anchor filaments and t-tubules. The proportion of cell exterior membrane (ES face) and cell interior fracture faces were similar to control tissue but, extensive small "en face" patches of PS (cytoplasmic) face of the plasmalemma were exposed (Fig. 2 a,b). In addition, the nuclei were enlarged, and showed a clumped chromatin pattern. These changes were confirmed by TEM (Figs. 2 c,d).

When control tissues were sliced under ethanol, disrupted and fractured myofibrils were frequently seen separating from the PS (cytoplasmic) face of the plasmalemma and focally exposing it (not illustrated). Ischemic tissue samples sliced under ethanol showed similar exposure of the plasmalemmal PS (cytoplasmic) face to that noted in control myocardium.

PS Face Appearance

The "en face" portions of the PS (cytoplasmic) face of the plasmalemma were easily identified in longitudinal fractures or slice exposures because the

raised periodic Z folds (Fig. 2 a,b) of the plasmalemma were characteristic of this surface. These folds are the site where the plasmalemma attaches to the Z line of subjacent myofibrils. The portion of the PS face between the Z-fold sites typically is separated from the myofibrils by a single mitochondrion.

The PS face exhibited four additional noteworthy structural features. First, there often was a partial longitudinal (typically half step) shift in the alignment of Z folds within individual cells. This is illustrated in Figs. 2 a,b; 3, 4; the angulated interconnection of the displaced Z folds is particularly obvious in Fig. 3. Second, round, elevated stump-like tubules about 100-150nm in diameter were seen in parallel rows at the level of the Z folds (Fig. 4). These are considered to be the sheared ostia of plasmalemmal invaginations (T-tubules). The third feature is a complex network of anastomosing cord-like structures 75-150 nm in width (Figs. 4-5) distributed between Z folds and showing a degree of radial orientation about T-tubule ostia. These cords generally had a parallel orientation to the Z folds. From SEM alone, it was impossible to determine whether these were solid or tubular. Interspersed between the cord-like array was the fourth structural component, a dense population of irregularly rounded nodular or dome shaped structures (Fig. 5a,b). While some were contiguous with the cord-like network, others were clearly separate. The diameter of these nodules varied from 70-200nm. No filamentous array of lesser diameters than 50nm could be demonstrated consistently on the PS face.

Although the integrity of the bond between PS face and the myofibrillar Z band could not be tested in samples sliced under ethanol (Sommer and Waugh (1976) technique) because of the high probability of mechanical disruption artefact, this bond was examined in fractured blocks. In both control and ischemic samples the adherence was observed to be an "all or none" phenomenon, with the myofibril attached at each contiguous Z band or completely detached. This is illustrated in Fig. 6.

The differences in appearance of control and ischemic PS plasmalemma faces were of degree only. Figs. 5a,b show typical appearances. The ischemic samples showed longer sarcomere periodicity relative to control and there was an apparent loss of density of both nodular and cord-like structures. Z folds in ischemic samples were thinner and the capping material seen in Fig. 5a cresting the Z folds was considerably less prominent in ischemic samples.

Quantitative Changes

The prevalence of PS (cytoplasmic) face seen "en face" on fracture faces of control myocardium ranged from 0-9.6% with a mean of 0.70% (SD, 1.22). After 40min of in vivo ischemia, the proportion ranged from 0-17.5% with a mean of 5.01% (SD, 3.72). Pooling the values (n = 2x120) or retaining separate samples (n = 12x20), the differences in means were statistically significant ($p<0.005$).

Preparative Differences

No additional structures were resolved using the osmium-ligand method but these procedures had some advantages. First, the myofibrils were more stable and could be imaged at 20-30kV for prolonged intervals without the bowing distortion of sarcomeres and angular crimping of I bands which occurs commonly in non-osmicated samples (Fig. 6). Similarly, cell membranes at both plasmalemma and organelle loci of the osmicated tissue were considerably more stable to the electron beam. All three osmium ligand methods provided similar results though the GTA-O-TCH-O method was preferred because this procedure took the least time for preparation. All three methods resulted in much more brittle tissue blocks with the result that shattering was common during freeze-fracture of small samples. Although specimen charging was suppressed by all three methods, the best resolution resulted from addition of a thin sputtered coat of gold-palladium to osmicated samples.

Figure 3: SEM of plasmalemmal PS face of an ischemic cardiac muscle cell. Organelles are detached except for a few swollen mitochondria (m) and the longitudinal shift in alignment of Z-folds (Z) of 1/2 - 1/3 sarcomere length is clearly evident at center. Bar = 1 µm.

Figure 4: SEM of control cardiac muscle showing mitochondria (m) and myofibers (f), a raised longitudinal plasmalemmal fold (broad arrow) and at right a large area of PS face. Z-folds are seen as prominent ridges (Z). The 3 stump-like ostia (fine arrows) are sheared t-tubules. The PS face shows typical anastomosing cord appearance, similar to that overlying the myofibrils (left) and probably consisting largely of SR. Bar = 1 µm.

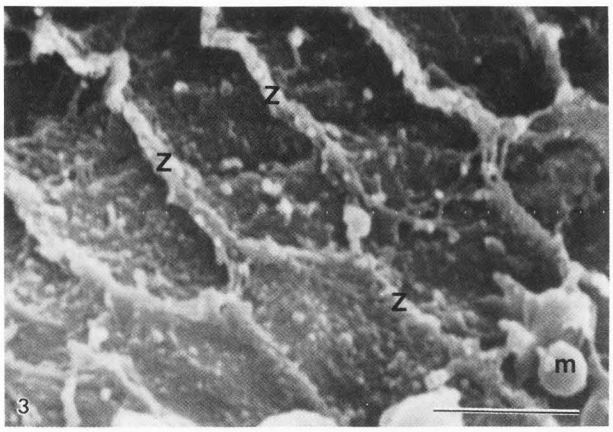
Discussion

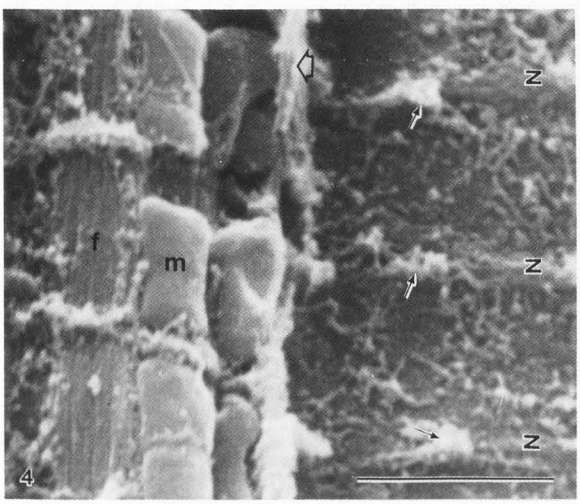
We used SEM to investigate the changes occurring in dog cardiac myocytes damaged by 40 minutes of severe ischemia in vivo. In order to test the hypothesis that cytoskeletal alteration precedes plasmalemmal damage, we analyzed carefully the region of attachment of the cell membrane to the underlying myofibrillar apparatus. Previous studies using SEM have examined cardiac membrane systems and their related invaginations the t-tubules (McCallister et al., 1974; Ashraf & Sybers, 1975; Ashraf et al., 1976; Forbes & Sperelakis, 1983; Ashraf, 1982), and the ES face of the sarcolemma with its surface lamina and intercellular collagen (Borg & Caulfield, 1981). "En face" views of the PS face of cardiac myocytes which should provide information about the membrane-myofibrillar attachment complexes, have only been illustrated (indistinctly) on one occasion (McCallister et al., 1974). No systematic SEM description of cardiac myocyte PS face has been published before though Sawada et al. (1978) have provided a short description and illustration of this feature of frog skeletal muscle.

Swelling of myocytes damaged by 40 minutes of ischemia produced the most striking changes seen by SEM. The relationship of the plasmalemma to subjacent organelles was changed two important ways. First, spaces were demonstrable between the PS (cytoplasmic) face of the plasmalemma (seen on edge) and the underlying myofibrils or mitochondria. This occurred over the central region of each sarcomere but did not involve the membrane-myofibril attachment at the Z-band complexes. Secondly, the prevalence of PS (cytoplasmic) face of the plasmalemma seen "en face" in fractured blocks increased significantly.

Myocyte Swelling

Myocyte swelling is indicative of the gain in cell water from the extracellular space partially compensating for increased cell osmolarity which results from accumulation of glycolytic products, principally lactate, and catabolism of high-energy phosphate reserves to smaller end products during ischemic injury (Jennings and Reimer, 1981; Tranum-Jensen et al., 1981). Following fixation and critical point drying, such swollen cells showed spaces between organelles representing removal of highly hydrated cytoplasm in the drying process, whereas control myocytes showed a compact planar interior (Sage and Gavin, 1985; 1986).





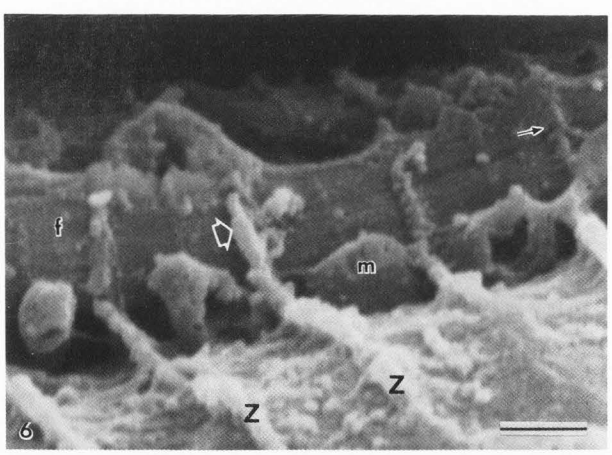
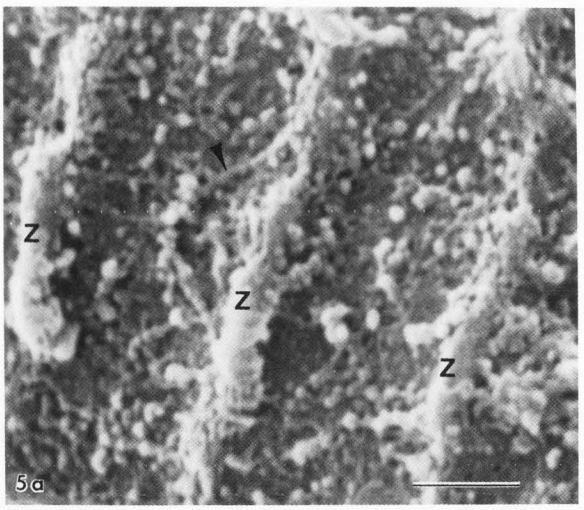


Figure 6: SEM of ischemic muscle cell PS face showing a single myofiber (f) attached to each Z-fold (Z). Z-line crimping of the myofiber (fine arrow), and broad cords overlying Z-lines corresponding to t-tubules (broad arrow) are seen. Remnants of swollen mitochondria occupy each subplasmalemmal space (m). Bar = 0.5 μm.



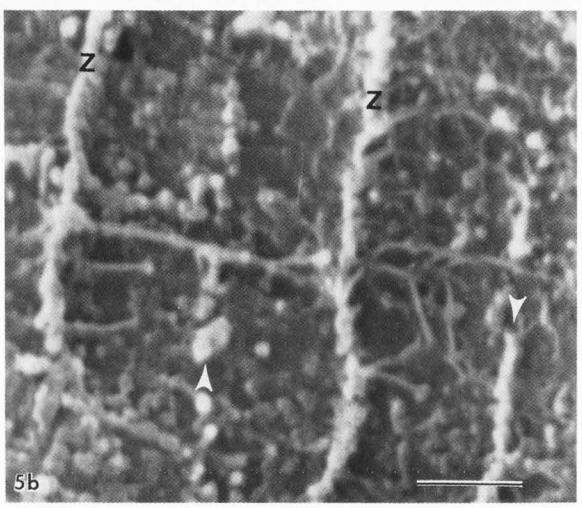


Figure 5a: SEM Detail of the PS face of control cardiac muscle plasmalemma showing Z-fold ridges (Z) and intervening, a dense array of anastomosing cords 70-100 nm wide (arrow head) and domed nodules or granules 100-200 nm in diameter. Bar = 0.5 μm.

Figure 5b: SEM detail of PS face of cardiac muscle plasmalemma after 40 min ischemia in vivo. Z-bands (Z) are thinner than in control and the cord/nodule array appears less dense, with some flat featureless areas appearing. A definite alignment of structures parallel to and midway between Z-bands is seen (arrow heads). Bar = 0.5 μm.

The present study utilized dog papillary muscle either uniformly severely ischemic, or control muscle known to be non-ischemic. Although spatial and temporal evolution of ischemic injury in dogs in vivo following coronary artery ligation varies markedly according to the intrinsic degree of collateral blood flow (Reimer and Jennings, 1979), the widely used protocol of left circumflex artery ligation virtually ensures severe ischemia of the PPM (Jennings and Wartman, 1957); moreover, blood flow measurements were made with microspheres to confirm that the samples studied were from areas equivalently injured by very severe ischemia.

The subplasmalemmal spaces described here are not "blebs" as previously described by TEM (Jennings et al., 1978) in dog hearts after longer intervals of severe ischemia in vivo, because "blebs" span several sarcomeres and this requires dehiscence of myofibrillar-membrane attachment at at least one Z-band. Such blebs frequently can be seen by SEM and TEM at later ischemic intervals (unpublished results), but were not observed in the 40 minute ischemic samples of this study. Z-band level myofibril-membrane attachment complexes remained intact, as did the contiguous attachments at Z-band level between myofibrils deeper in the cell.

The PS (cytoplasmic) face of the plasmalemma

A key finding of this study was the significant increase in prevalence of "en face" portions of the PS (cytoplasmic) face of the plasmalemma in freeze-fracture faces of ischemic papillary muscle. Its prevalence in control tissue is low (0.70%), but is considerably greater than that observed for the intramembranous PF and EF faces (<0.01%) the plasmalemmal membrane. Robards and Sleytr (1985) have previously commented that the use of dehydrated fixed tissue changes the prevalence of the intramembranous fracture planes which are the predominant surfaces seen in many TEM freeze-etch studies of fully hydrated unfixed tissue.

Two possible explanations can be considered for the rise in prevalence of "en face" PS face membrane in ischemic fracture planes. First, the induction by cell swelling of the highly hydrated subplasmalemmal spaces described above may have created a plane of low shear resistance in the subplasmalemmal space not present in control myocytes. The freeze-fracture plane, which follows the path of lowest shear resistance, would then expose the PS face with a prevalence related solely to cell swelling and the creation of subplasmalemmal spaces.

Alternatively the increased prevalence of "en face" PS face appearance could reflect weakening of attachment of the plasmalemma to underlying myofibrils. In a recent study of in vitro ischemia of dog myocardium (Steenbergen et al., 1987) it was shown that the antigenicity of one of the components of membrane-myofibril attachment complexes (vinculin) is lost during the early phase of irreversible ischemic injury. In the present study, we could not demonstrate by SEM any structural changes in the integrity of membrane-myofibril attachment after 40 min in vivo ischemia, though Z-folds had less substantial capping material and appeared thinner. It may be that alterations to attachment complexes (or to other cytoskeletal components sited between Z-bands) too small to be resolved by the sample-instrument combination of this study had occurred. Indeed the appearance of subplasmalemmal spaces themselves may point to a loss of cytoskeletal components in the subplasmalemmal space between rather than at Z-bands, consistent with the hypothesis that such alteration precedes membrane damage (Jennings et al., 1986), but this remains unproven in this study.

The five structural components of the PS face described in this paper were interpreted with additional data from parallel TEM studies, as surface images cannot always distinguish solid from tubular structures. From the thin section images it is suggested that the anastomosing network evident between Z folds is composed predominantly of peripheral junctional sarcoplasmic reticulum (nomenclature: Sommer & Jennings, 1986). Similarly the nodular-granular appearance can be accounted for by membrane caveolae, glycogen granules, ribosomes and SR components adherent to the PS face.

The ordered orientation of the structures parallel to the Z folds with spacing corresponding to the M band of overlying myofibrils has received little comment in cardiac muscle previously. As the M band and the Z line are the effective endplates of the sliding filament contractile apparatus of the sarcomere, they are the only structures at which fixed length cytoskeletal components could bridge between membrane and myofibrils. Though M line - membrane bridges have been described in skeletal mucle (Peirobon-Bormioli, 1981) and inferred by others (Shear and Bloch, 1985), we have been unable to demonstrate similar attachments at the M band level in thin sections (TEM) of these samples of cardiac muscle, even in sarcomeres lacking subplasmalemmal mitochondria. It is likely that the structures seen in SEM micrographs represent, at least in part, elaboration of peripheral junctional SR at the A band as suggested by Shear and Bloch (1985).

Skeletal muscle is recognized as showing a more regular myofibrillar array (Fibrillenstruktur) than cardiac muscle (Feldstruktur), while the frequent lateral branchings of cardiac muscle cells are absent from skeletal muscle (Sommer and Jennings, 1986). It is not surprising therefore that there should be shifts in alignment between sarcomeres of adjacent myofibrils in cardiac myocytes and of overlying Z fold periodicity on the PS face of cardiac plasmalemma. Nor is it surprising that these shifts have not been observed previously in studies of skeletal muscle. Tokoyasu (1983) has described the shift in periodicity of sarcomeres in adjacent myofibrils of chick cardiac muscle, but the changes on the overlying plasmalemma do not appear to have been previously described. This shift may have practical relevance in limiting the interpretation of dark-field fluorescent light microscopic immunolocalization of myofibril-membrane attachment complex antigens, and it may imply differences in the structural and functional character of attachment complexes in the two types of striated muscle.

The detectable morphological differences in ischemic myocardial PS face compared to control were relatively minor. The increased sarcomere length reflects the expected relaxation of myocytes following 40 min ischemia, and the decreased density of surface structures may be accounted for by extension of the membrane (as a function of myofibrillar relaxation) and catabolism of glycogen granules in the subplasmalemmal space. Relatively high gun voltages were required to resolve fine detail in this study, and the resulting ability of the rastered electron beam to produce artefactual holes in the PS face, even after osmium-ligand preparation, meant that it was not possible to confirm by SEM the TEM findings of appearance of plasmalemmal (but not necessarily surface lamina) defects by this stage of ischemic injury (Jennings and Reimer, 1981).

In summary, we demonstrate in this study that dog cardiac muscle subjected to severe in vivo ischemia of duration sufficient to result in irreversible injury, retained structurally intact membrane-myofibrillar attachments at Z-band level, despite marked cell swelling. The increased prevalence of PS-face exposure by freeze-fracture may indicate a functional alteration to Z-band level attachments. This finding suggests that if ischemic damage to cytoskeletal structures is a predisposing step in plasmalemmal injury, then these alterations may occur first at sites between rather than at Z-band level. Though we give a clear SEM

description of the PS-face of the plasmalemma in control and ischemic myocardium, the determination of changes to any putative subplasmalemmal fibrillar cytoskeletal network at A- and I-band levels of the sarcomere was beyond the resolution of the sample-instrument combination of this study, and is the subject of further investigation.

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Discussion with Reviewers

M. Ashraf: The subsarcolemmal spaces contain numerous granular/nodular structures (on PS face). It is likely they are SR, glycogen and other structures. The authors can clearly confirm them by embedding the specific SEM fields from the SEM samples for TEM examination.

T. Borg: Did the authors do any TEM on the samples that had been previously examined by SEM?

Authors: TEM of SEM samples was not successful. We confirmed the finding of Murphy (1980) that GTA-O-TCH-O samples show considerable hardening and were too brittle to thin-section satisfactorily. The repeated prolonged cycles of osmication are not ideal for preservation of any putative subplasmalemmal

cytoskeleton (vide infra), so that further studies have used other preparation techniques.

T. Borg: The Branton et al. terminology was written for TEM freeze fracture replicas not freeze cleaved SEM samples. Is this terminology appropriate for these SEM images?
Authors: Freeze-cleaved SEM samples are in effect "infinitely etched" (completely dried) rotary replicas, from which it is unnecessary to digest the tissue substrate for viewing by SEM. Though cleavage of fixed frozen blocks may result in fracture plane location differences compared with freeze-fracture of unfixed tissue in proprietary devices, the same descriptive terminology is still appropriate. The Branton et al (1975) nomenclature (text ref. 5) has been widely adopted for TEM replicas because it imposed a simple uniform system on what had become a semantic quagmire of prolix descriptive terms. The brief mnemonics allow unequivocal identification of membrane surfaces - an essential feature in this paper where the true inner, or cytoplasmic surface (PS face) is the plane of interest.

T. Borg: Was there any evidence of regional swelling in individual myocytes or was it uniform along the entire surface? Do regional differences relate to the location of extracellular matrix components such as collagen?
Authors: After 40 min ischemia in vivo swelling was extensive but not uniform within individual cells. Presence or absence of swelling does not relate directly to location of vessels, major collagenous bands or to cell locus within myocyte bundles. We have unpublished data indicating that this is also true of total ischemia in vitro for 30-180 min in dog papillary muscle.

T. Borg: Is there any immunohistochemical evidence as to the identification of the microfibrillar structures on the cytoplasmic side of the sarcolemma?
Authors: Immunohistochemical localization for α-actinin, vinculin and desmin has been unsuccessful in our hands using methods of periodate-etched Epon-embedded or LR gold-embedded rat myocardium fixed in glutaraldehyde or paraformaldehyde (Bendayan and Zollinger (1983) J. Histochem. Cytochem. 31: 101-109). However, we have data currently prepared for publication indicating the presence of a previously unreported periodic cortical subplasmalemmal microfibrillar array in dog papillary muscle fixed with aldehyde-amine solutions at 37°C, and subjected to reduced periods of osmication. Further study utilizing Protein-A gold immuno-EM on ultrathin frozen sections is in progress.

J. Caulfield: If the plasma membrane is broken in irreversibly ischemic heart, how do the cells remain swollen?
Authors: Myocyte swelling is induced during ischemia by the production of osmotically active catabolites from high energy phosphate reserves. After the plasma membrane is broken, osmotic equilibrium will be attained, but no reverse gradient to restore original cell volume is generated. There appears to be insufficient elastic force in the ischemic sarcolemma to return it to its uninjured dimensions, even after osmotic equilibrium is reached.