SURFACE FEATURES OF STRIATED MUSCLE

I. GUINEA-PIG CARDIAC MUSCLE

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SUMMARY

A general survey of guinea-pig myocardium was undertaken using the freeze-etch technique. Replicas of myocardial cell membranes were obtained. These showed an ordered array of pits or stumps situated at Z levels. The pits are interpreted as the apertures of the transverse tubules (T-tubules) seen from outside the cell, and the stumps as the remnants of the T-tubules remaining attached to the cell membrane after the cell contents have been removed. Pinocytic vesicles were also present. T-tubules, mitochondria and myofilaments could be seen in replicas of the interior of myocardial cells.

Capillary endothelial cells were seen from various aspects; pinocytic vesicles were their most prominent feature.

The appearances of the cell membranes in the present study suggest that the fracture plane tends to pass along either the outer or the inner surface of the membrane rather than to split the membrane.

INTRODUCTION

The technique of freeze-etching, a process originated by Steere (1957) and fully described by Moor & Mühlethaler (1963) and Moor (1964) has been applied to various tissues, including mammalian myocardium, by Moor, Ruska & Ruska (1964). When the frozen tissue is being fractured, the plane of cleavage tends to follow lines of least resistance; in particular, it often follows the cell membranes so that quite large areas of these may be exposed to view. Using this method, the present authors have investigated guinea-pig heart muscle with specific reference to surface features of the plasmalemma and to the T-tubule system. Some of the findings have already been briefly reported (Rayns, Simpson & Bertaud, 1967).

MATERIALS AND METHODS

Guinea-pigs were subjected to thoracotomy under ether anaesthesia. Three ml of Ringer solution containing 30 % glycerol were injected into the still-beating heart and after a few minutes the heart was opened and small pieces of papillary muscle were dissected out. These were then mounted on small copper discs, rapidly frozen to −150 °C by immersion in liquid Freon held just above its melting point and transferred to a Balzers BA 500R Freeze-Etch apparatus. The fracturing, etching,
shadowing and replication techniques used were essentially as described by Moor & Mühlethaler (1963) and Moor (1964). The replicas were examined in a Philips EM 200 electron microscope.

RESULTS

Replicas were obtained of the surfaces and contents of myocardial cells, and these form the main basis of this report. Replicas of capillary endothelial cells were also obtained and will be discussed briefly below ('Capillaries').

Surface features

*Invaginations.* Surfaces of myocardial cells were frequently seen in replicas of favourably fractured material. In some cases, the surfaces were transversely creased (e.g. Fig. 3, upper region), the spacing between the creases being about 1.4 µ and thus presumably corresponding to sarcomere length. This creasing was interpreted as evidence of the cell in question having been in a contracted state at the time of freezing. More usually the cell surfaces were not creased to any marked degree (e.g. Fig. 3, lower region; Figs. 1, 2, 4, 7) and had presumably belonged to cells which were not greatly contracted.

The surfaces always showed one of two characteristic appearances. On the one hand, some surfaces (e.g. Figs. 1, 2, 3 upper region) showed an array of round or oval depressions. (A depression can be identified by the heavy metal deposit on the side farthest from the shadowing source.) These depressions were arranged in roughly parallel rows in both the transverse and the longitudinal directions. The longitudinal spacing was about 1.4–2.0 µ while the transverse spacing was usually a little larger and rather more variable. Replicas showing this appearance were interpreted as representing the outer surface of a myocardial cell membrane, and the depressions in the surface were interpreted as the apertures of the T-tubules.

On the other hand, some surfaces (e.g. Fig. 3, lower region; Figs. 4, 7) showed round or oval structures, arrayed in a similar manner to the apertures mentioned above but clearly projecting upwards from the surface. (An upward projection can be identified by the dense deposit of heavy metal on the side nearest to the source of shadowing and by the pale or negative shadow cast beyond the projection.) Replicas showing this appearance were interpreted as representing the inner or cytoplasmic aspect of the myocardial cell membrane, the projections being the stumps of the transverse tubules broken off near the cell membrane.

The number of T-tubule apertures (or stumps), and the regularity of their array, varied considerably in different cell-surface replicas. In some instances the array was very regular while in other cases quite large areas of membrane seemed to contain no apertures (e.g. part of Fig. 7).

It was not feasible to estimate the heights of the T-tubule stumps from the lengths of their shadows because of the undulating nature of the surface. The broken ends of the stumps were usually oval, with the longer diameter oriented transversely to the long axis of the cell, and this longer diameter varied from 0.15 to 0.33 µ. The shorter
Myocardial surface features

diameter varied from about 0.05 to 0.23 μ. This variation in diameter probably in part represents a real difference in tubule size and in part is a function of how close to the surface the tubule was broken. In sectioned material (e.g. Simpson & Oertelis, 1962; Simpson, 1965) the mouths of the T-tubules appear somewhat trumpet-shaped, and therefore the broken ends of a tubule will appear widest if the break has occurred very close to the mouth of the tubule.

In addition to these features representing T-tubule apertures, the myocardial cell surfaces also contained smaller round structures, 500–800 Å in diameter, which were interpreted as pinocytotic vesicles (Fig. 2). These were irregularly distributed in longitudinal rows, often along the rows occupied by the T-tubule apertures.

It was not always easy to interpret all the features seen on the external aspect of a cell surface, as in Fig. 1, where many irregularities are present and where only some of the T-tubule apertures are clearly seen as depressions in the surface; other T-tubule apertures in this case appear to contain a rounded plug of material.

Particles. A close examination of the plasmalemma revealed the presence of numerous small spheroidal particles 75–100 Å in diameter (e.g. Figs. 2, 4). These were randomly distributed over all plasmalemmal surfaces examined. From counts made on numerous micrographs the distribution of these particles was investigated. The population density was found to be 80–120/μ² on surfaces where the T-tubule apertures appeared as stumps and 400–700/μ² on surfaces where these features appeared as depressions. They were also observed on endothelial cell membranes (Fig. 9). Similar particles have been reported for other cell membranes (Staehelin, 1966).

Internal features

Transverse tubules. Tubular structures were visible in surface view lying between the groups of myofilaments. These were interpreted as elements of the transverse, or T-tubule, system (t, Figs. 5–7). Figure 6 shows the different aspects of longitudinally fractured tubules that can be encountered. At w the intact tubule is shown lying partly above the general replica surface; here the surface presented by the tubule is believed to be the inner (cytoplasmic) surface of the plasmalemma. At x the tubule has been fractured in various ways, revealing its contents (i.e. the extracellular fluid). In the adjacent areas, y, the tubule contents have been completely removed, revealing the outer (non-cytoplasmic) membrane surface, i.e. the external surface of the plasmalemma. Branching of these tubules was evident in some replicas with arms running both transversely and longitudinally (t₁, t₂, Fig. 7, and t₃, Fig. 6). Occasionally there were tubular elements of much smaller diameter than the T-tubules lying in close association with the myofilaments. In the few cases observed, these ran more or less parallel to the myofilaments (r, Fig. 6) and they are believed to be parts of the sarcoplasmic reticulum. The relationships between the T-tubules within cells and the apertures at cell surfaces could be seen in favourable replicas (e.g. Fig. 7).

Myofilaments. The majority of cells examined had been fractured obliquely to the myofibrillar axis. Such cells (Figs. 5, 6) revealed a number of general internal features. There were numerous groups of small circular profiles (f) each containing a central electron-dense dot and showing a white shadow; these are the ends of the thick myosin
filaments. Because of the etching procedure each myosin filament projects about 200 Å above the surrounding matrix and if favourably oriented with respect to the shadowing source receives a coating of platinum–carbon on one side. It is this metal layer, seen more or less end-on, which gives rise to the central black dot. In addition, each upstanding filament becomes evenly coated with a layer of carbon about 200 Å in thickness; this seen end-on gives the appearance of a halo of intermediate density. In some places the hexagonal array of the filaments could be observed. Similar views of the myofilaments have been published by Moor et al. (1964).

Mitochondria. Lying between the groups of myosin filaments were the mitochondria, \textit{m}. In these replicas an individual mitochondrion may exhibit one of three different appearances (Fig. 5): first, a slightly rough convexity standing above the general surface level, \textit{ms}, representing part of the outer surface of a mitochondrion of which the main bulk lay below the specimen surface; secondly, a rounded hollow, \textit{mh}, where a whole mitochondrion has been removed; thirdly and most commonly, fractures of the mitochondrial interiors, \textit{m}, which clearly reveal the cristae. Such internal aspects of mitochondria are seen also in Figs. 6–8.

Intercalated discs. In both oblique and longitudinal fractures which passed through intracellular junctions one could find apposed cell borders following sinuous courses across the replicas (Fig. 8). These probably represent intercalated discs or portions of surface membranes close to an intercalated disc.

Capillaries
Various aspects of blood capillaries were seen in the replicas. In oblique fractures (Fig. 5) it was possible to see both the substance of the endothelial cells and their inner and outer surfaces. Views of the two cell surfaces—that is, the surface apposed to the vessel lumen and the surface adjacent to the extra-capillary space—showed numerous small shallow depressions, some of which appeared to be filled with extracellular material. With the techniques employed here it was not possible to demonstrate a basement membrane associated with either the endothelial cells or with the muscle cells. In the cell substance there are clearly discernible vesicles in communication with the extracellular space (small arrows, Figs. 5, 9); the apertures of these vesicles were of a similar size to those depressions seen in the membrane surfaces and they were considered to be the pinocytotic vesicles.

Where the fracture plane had passed along the length of a capillary, striking views of endothelial cell membranes were obtained. In Fig. 9, for example, the fracture has passed along the inner face of the plasmalemma adjacent to the capillary lumen, demonstrating the generally undulating surface of the cell, which bears numerous small rounded features of two types. First, there are those which appear as rounded objects raised above the general surface, and secondly, there are those with raised rims and sunken centres resembling minute volcanic craters. These profiles are interpreted as pinocytotic vesicles. In the first type the membrane depressions were presumably relatively shallow, so the fracture plane passed around them, leaving the vesicles intact, whereas in the second type rather more deeply indented vesicles have been removed, leaving only the apertures plugged with extracellular material.
DISCUSSION

It is well established that tubules of the transverse or T-system of mammalian myocardial cells are in direct communication with the extracellular space via sarcolemmal invaginations (e.g., Simpson & Oertelis, 1961, 1962; Nelson & Benson, 1963; Simpson, 1965). In frozen-etched surface replicas (Figs. 1–4, 7) the apertures of these invaginations can readily be seen, either as pits in the surface membrane or as stumps projecting from it. It seems reasonable to deduce that the replicas showing these appearances represent respectively the outer (non-cytoplasmic) surface of the plasmalemma and the inner (cytoplasmic) surface (Rayns et al., 1967). Thus, examination of the inner surface (seen from within the cell) would reveal the invaginations standing upwards but broken off near their bases and appearing as short stumps casting white shadows across the membrane (Figs. 3, 4, 7). Conversely, examination of the plasmalemma from outside the cell would reveal the apertures of these invaginations as sunken pits casting no such shadows (Figs. 1, 2).

The dimensions of these structures seen in the surface replicas, their array, and the distances between them all bear out the interpretation that it is the apertures of the T-tubules which have been demonstrated. The freeze-etching technique has therefore made it possible to obtain an idea of the distribution and frequency of T-tubule apertures. The present results in this respect are only preliminary, but they seem to show that in some regions the array of apertures is extremely regular, with an aperture at each point where one could be expected, while in other places (e.g., Fig. 7) there are areas of membrane with unexpectedly few apertures.

The nature of the membrane surfaces exposed in freeze etching is still under discussion (Branton, 1966; Moor, 1966; Branton & Park, 1967; Deamer & Branton, 1967; Staehelin, 1968 and personal communication). Branton & Park (1967) have shown most convincingly that in chloroplast membranes cleavage takes place along a central plane so that the true outer surfaces are not seen. Their results appear to support the hypothesis that chloroplast membranes are based on an array of lipoprotein subunits (Green & Purdue, 1966; Benson, 1966) rather than on a central lipid bilayer (Robertson, 1959). Deamer & Branton (1967) have demonstrated by means of radioactive tracer experiments that frozen artificial phospholipid bilayers may also be split along their midplanes.

Staehelin (1968), on the other hand, has shown that when artificial bilayers of phospholipid are subjected to normal freeze-etching procedures they undergo only a partial splitting, which leaves a considerable proportion of the area of the upper leaflet as islands adhering to the non-polar surface of the lower. He has also shown that many natural membranes, including the plasmalemma of various cells, may sometimes present a continuous particle-covered surface, and at other times undergo a partial splitting similar to that which occurs in artificial bilayers. In the latter cases the particles appear on the islands of the upper leaflet rather than on the inner surface exposed by splitting. His results indicate that the plasmalemma and some other cellular membranes do have as their central component a lipid bilayer structure, and that in these membranes the particles are distributed over the true outer surface.

If the plasmalemma were to be split completely, this would seem to require that
when it is viewed as from inside and outside the cell the two surfaces should on the average fit one another, the particles on the one matching in number and distribution the pits on the other. In the present work, muscle cell surfaces consistently display one of two distinctive appearances, depending on whether they are seen from inside or outside the cell, but they certainly do not match one another as though formed by splitting. They differ chiefly in their particle population, one having on the average five times as many as the other. Neither shows any pattern of pits which could correspond with the distribution of particles on the other. These facts, together with the evidence presented by Staehelin concerning the plasma membrane surface in other types of cells, lead to the conclusion that the surfaces shown in Figs. 2 and 4 are most probably the outer and inner surfaces respectively of the plasmalemma, i.e., the membrane itself has not been split. Mühlethaler, Moor & Szarkowski (1965) have suggested that the surface particles may be enzymic in function and concerned with the physiological activity of the membrane.

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REFERENCES


**Myocardial surface features**


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**PLATES**

In each figure the double-headed arrow indicates the direction of metal shadowing.

**ABBREVIATIONS ON PLATES**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>a</td>
<td>aperture (invagination) of T-tubule</td>
</tr>
<tr>
<td>Ab</td>
<td>A-band of sarcomere</td>
</tr>
<tr>
<td>e</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ex</td>
<td>extracellular space</td>
</tr>
<tr>
<td>f</td>
<td>myosin filaments</td>
</tr>
<tr>
<td>Ib</td>
<td>I-band of sarcomere</td>
</tr>
<tr>
<td>id</td>
<td>intercalated disc</td>
</tr>
<tr>
<td>l</td>
<td>lumen</td>
</tr>
<tr>
<td>m</td>
<td>mitochondria</td>
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<tr>
<td>p</td>
<td>plasmalemma</td>
</tr>
<tr>
<td>pi</td>
<td>inner surface of plasmalemma</td>
</tr>
<tr>
<td>po</td>
<td>outer surface of plasmalemma</td>
</tr>
<tr>
<td>pv</td>
<td>pinocytotic vesicles</td>
</tr>
<tr>
<td>r</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>s</td>
<td>stumps of T-tubules</td>
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<tr>
<td>t</td>
<td>T-tubule</td>
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Fig. 1. Semi-longitudinal fracture, showing parts of two myocardial cells. $\times 7200$.

At left centre, the plasmalemma of one cell (po) is viewed from outside the cell, and rows of apertures of transverse tubules can be seen ($a_1$, $b_1$, $c_1$). In some cases these apertures appear as true pits (e.g. $a_4$, $c_4$) but in most cases they appear to be 'plugged' with some material. Some members of the array of apertures seem to be missing, e.g. $c_8$, $c_5$, $c_6$, $b_6$, while $a_4$ and $a_8$ appear to be fused.

At upper right, the interior of the same cell is seen fractured obliquely. At the bottom of the figure the interior of another cell is seen. Towards the right, the cell contents have broken away from the plasmalemma, thus exposing its inner surface (pi) on which can be seen the stump of a broken-off transverse tubule ($i$).

Fig. 2. Outer surface of plasmalemma, showing apertures of T-tubules, are, ax and ay, and also numerous variously shaped depressions, po, probably representing stages in pinocytosis. When present, these depressions tend to be gathered in longitudinal rows, sometimes coinciding with the longitudinal rows of T-tubule apertures (left centre). $\times 35300$. 
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(Facing p. 474)
Fig. 3. Extensive areas of the surface membranes of two myocardial cells are seen, separated by a small extracellular space, ex. The myofibrillar axis lies from upper left to lower right. The upper cell, which presents the outer surface of the plasmalemma, po, is partially contracted and shows transverse creasing of the surface in the regions of the invaginations (a); the lower cell presents the inner surface of the plasmalemma, pi, and is in a more relaxed state with little creasing evident. When the apertures of the transverse tubules are viewed from the outside of the cell (upper) they appear as depressions in the surface (a); when they are viewed from inside the cell, they appear as short stumps (s) projecting from the plasmalemma. There is clearly an ordered array of the invaginations in the longitudinal and transverse directions in both cells. The two rows of stumps sa and sb converge at sc to become a single row. At the lower left corner, a blood capillary is seen from various aspects. × 8300.

Fig. 4. Part of a cell membrane showing the inner surface of the plasmalemma, demonstrating the stumps of the T-tubules, s, standing erect in both longitudinal rows and transverse rows. Situated on the more or less smooth surface are randomly scattered small spheroidal particles (small arrows). The myofibrillar axis lies vertically. × 55400.
Fig. 5. An oblique fracture of two myocardial cells and part of a blood capillary lying between them. Within the myocardial cells are some cut ends of myofilaments, $f$, and several fractured mitochondria, $m$. In the left-hand cell can be seen the surface of a mitochondrion, $ms$, and one depression, $mh$, left by a mitochondrion removed in the fracturing process. In addition, there are parts of two T-tubules, $t$, lying between the myofilaments. The capillary has been fractured in such a way that not only the substance but also the outer and inner surfaces of the endothelial cells, $e$, are visible. Pinocytotic vesicles, $pv$, are numerous. Some of these vesicles communicate directly with the extracellular space (small arrows). $\times 21600$. 
Fig. 6. An oblique fracture of a myocardial cell, showing in particular the course of the T-tubules (t₁ and t₂) in relation to the A-band (Ab) and the I-band (Ib) regions of the muscle. Tubule t₂ exhibits branching, showing both transverse and oblique or longitudinal elements. In both tubules t₁ and t₂ the cytoplasmic surface of the tubule membrane may be seen at w, the tubule contents at x, and at y a depression remaining after the tubule contents have been removed by the fracturing process. This depression is lined by the non-cytoplasmic surface of tubule membrane. × 19800.

Fig. 7. Longitudinally fractured myocardium, demonstrating the interior of a cell and the inner surface pi of the plasmalemma of the same cell. Within the cell substance, besides the mitochondria, m, and longitudinally oriented myofilaments, f, can be seen transverse elements of the T-tubule system t₁–t₄ (t₄ is not in the plane of fracture). A longitudinal connexion exists between tubules t₅ and t₆. On the plasmalemma are the apertures of the T-tubules, t₁–t₆, which show a degree of topographical relationship in the transverse direction, with the internal tubules t₁–t₆. × 17000.
Fig. 8. A longitudinal fracture of parts of three myocardial cells, showing A-bands (Ab) and I-bands (Ib) and mitochondria, m. An intercalated disc, id, runs across the centre of the micrograph. × 29 600.

Fig. 9. A longitudinal fracture, showing a capillary situated between two myocardial cells. The fracture has exposed mainly the cytoplasmic surface (pi) of that portion of the endothelial cell plasmalemma which lies adjacent to the vessel lumen. This surface is covered with small globular structures which are interpreted as pinocytotic vesicles and with crater-like structures representing places where pinocytotic vesicles have been removed in the fracturing process. Views of pinocytotic vesicles opening to the extracellular space (small arrows) can be seen where the fracture has passed through the cell substance. Above the vessel, the outer surface of the endothelial cell is seen (po). × 22 100.