Isolated calcium-tolerant myocytes and the calcium paradox: an ultrastructural comparison

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The ultrastructure of calcium-tolerant myocytes isolated from adult rat ventricular myocardium is described, using thin section and freeze-fracture electron microscopy. Two distinct cell types are observed, rounded and rod-shaped. The former are damaged myocytes and superficially resemble the disrupted cells characteristic of the calcium paradox. Despite this resemblance however, the genesis of these isolated damaged cells is not explicable in terms of the calcium paradox. The majority of isolated cells are rod-shaped and show well preserved ultrastructural features. Our results indicate that whatever mechanisms underlie the calcium paradox it is not an indispensable condition that isolation of myocytes necessarily leads to this phenomenon.

Introduction

The re-introduction of calcium at physiological concentrations to the coronary perfusate of hearts which have been exposed at 37°C to calcium-depleted solutions has catastrophic effects on myocardial ultrastructure[1-3]. However, in order to dissociate myocytes from adult heart tissue to provide individual cells in suspension, calcium-deficient solutions are required. It might therefore be predicted that the ultrastructure of isolated myocytes would also be severely disrupted following re-exposure to calcium-containing solutions.

Using thin section and freeze-fracture electron microscopy, we have investigated the ultrastructure of myocytes isolated from adult left ventricle by the in vitro collagenase-perfusion technique of Powell et al.[4]. Our results show that the population of rod-shaped myocytes obtained by this technique are well preserved ultrastructurally, closely resembling their counterparts in the intact myocardium.

Methods

ANIMALS

Hearts were obtained from adult female Sprague-Dawley rats (b.wt 200-300 g).

PERFUSION TECHNIQUES AND INDUCTION OF THE CALCIUM PARADOX

Isolated hearts were perfused at 37°C by the Langendorff technique[5] as described previously[6]. An initial 15-min equilibration perfusion with medium of the following composition was given: NaCl, 118.4 mmol l⁻¹; KCl, 4.75 mmol l⁻¹; CaCl₂, 1.8 mmol l⁻¹; MgSO₄·7H₂O, 1.19 mmol l⁻¹; NaHCO₃, 25 mmol l⁻¹; KH₂PO₄, 1.19 mmol l⁻¹; Glucose, 11.1 mmol l⁻¹. This was then replaced by the same medium but with calcium omitted. It should be emphasized that great care was taken to ensure that this solution and its associated glassware were calcium-free. Perfusion with the calcium-free medium was continued for 4 min, before reperfusion with the standard calcium-containing medium was carried out.

ISOLATED MYOCYTE PREPARATION

Suspensions of purified cardiac myocytes were obtained by Langendorff perfusion of rat hearts with crude collagenase in low calcium Krebs-Ringer bicarbonate buffer as described by Powell et al.[4].

The resulting myocyte suspension was preincubated at 37°C in gassed buffer containing 20 mg cm⁻³ bovine serum albumin for 30 min prior to experimental procedures. Preincubation was carried out in either nominally calcium-free buffer, or medium to which 0.5 mmol l⁻¹ CaCl₂ had been added. All myocyte suspensions were gassed regularly with 95% O₂-5% CO₂.
ELECTRON MICROSCOPY OF VENTRICULAR MYOCARDIUM

Hearts subjected to the calcium paradox were fixed by Langendorff-perfusion with 2% glutaraldehyde in 0-1 mol l⁻¹ sodium cacodylate buffer, pH 7.3 for 5 min at room temperature. Control hearts were fixed similarly, immediately after the 15 min equilibration period. Left ventricular tissue was removed and treated in the same fixative for a further 2 h. 1 mm³ samples were rinsed briefly in 0-1 mol l⁻¹ sodium cacodylate buffer and post-fixed in 1% osmium tetroxide buffered with 0-1 mol l⁻¹ sodium cacodylate buffer, pH 7.3 at 4°C for 2 h. After ‘en bloc’ staining with saturated aqueous uranyl acetate for 1 h at room temperature, the specimens were dehydrated in ethanol and embedded in araldite epoxy resin. Silver sections were prepared using an LKB III ultramicrotome and examined in a Philips EM 301 electron microscope.

ELECTRON MICROSCOPY OF ISOLATED MYOCYTES

Isolated myocytes were fixed for electron microscopy by adding 1 vol of myocyte suspension to 4 vol of 2% glutaraldehyde made up in 1 strength Krebs or cacodylate buffer. Myocyte suspensions were fixed for 2 h in the glutaraldehyde solution at 20°C, gently centrifuged to form a pellet and processed as described above.

FREEZE-FRACTURE OF ISOLATED MYOCYTES

Glutaraldehyde-fixed specimens were infiltrated with buffered 25% glycerol for 45 min. The cell suspensions were mounted on specimen holders and frozen by immersion in liquid propane at −180°C. Freeze-fracturing was carried out in a Balzers BAF 400T unit following standard procedures. Replicas were cleaned in 40% chromic acid before mounting for electron microscopy.

Results

Fig. 1 illustrates the typical ultrastructural features of myocytes in intact myocardial tissue from a control heart fixed after the 15-min equilibration perfusion period. After exposure to calcium-free solutions followed by re-introduction of standard calcium-containing medium, drastic ultrastructural damage is observed in the majority of cells (Fig. 2).

Figure 1  Control myocardium showing normal well-preserved features of myocyte ultrastructure. Note in particular, relaxed state of myofibrils alternating with rows of mitochondria, even distribution of chromatin in the nucleus (N) and close apposition of the sarcolemmata at the intercalated disc (ID). Scale bar, 10 μm.
Figure 2 Characteristic ultrastructural damage seen in rat myocardium subjected to the calcium paradox. Numerous contraction bands (C) are present, mitochondria (M) are aggregated, intracellular oedema is apparent and intercalated discs (ID) are disrupted. Scale bar, 10 μm.

Figure 3 Survey view of part of a rounded isolated myocyte. Extensive ultrastructural damage is evident: myofibrils have contracted into a central mass (F), with mitochondria (M) situated in oedematous and vaculated cytoplasm (O). Scale bar, 10 μm.
Myofibrils appear grossly contracted, intracellular oedema is evident and the sarcolemma is disrupted, especially at the intercalated discs.

On survey examination of isolated myocytes, two distinct cell populations are visible. The first population comprises rounded, damaged cells (Figs 3 and 4) which show ultrastructural features resembling those observed after the calcium paradox. Low magnification views (Fig. 3) show gross myofibrillar contraction, intracellular oedema and vacuolation. At higher magnification, mitochondria appear swollen with disrupted cristae (Fig. 4) as in the calcium paradox.

The second and more numerous cell type consists of rod-shaped cells (Fig. 5) which show all the gross morphological features characteristic of myocytes in intact tissue (Fig. 1). Inspection of these cells at higher magnification in both thin section (Fig. 6) and freeze-fracture (Fig. 7) confirms that the structural relationships between the mitochondria, tubular membrane systems and contractile apparatus are well preserved. Planar freeze-fracture views

**Figure 4** View of rounded isolated myocyte showing typical mitochondrial disruption with swelling and deformations of the cristae (C). Scale bar, 1 μm.

**Figure 5** Low magnification view of a rod-shaped isolated myocyte. Myofibrils are in regular arrays, mitochondria are distributed in rows and the nucleus reveals a normal appearance. No intracellular oedema is apparent. Scale bar, 10 μm.
Figure 6 View of the peripheral cytoplasm and nucleus of a rod-shaped isolated myocyte. Myofibrils, mitochondria and nuclear ultrastructure is well preserved. N, nucleolus; NP, nuclear pore. Scale bar, 1 μm.

Figure 7 Freeze-fracture image of a rod-shaped isolated myocyte showing a similar area to that depicted in Fig. 6. F denotes position of the myofibrils and M, mitochondria. Between the mitochondria, elements of the sarcoplasmic reticulum and transverse-tubule membranes are visible. Scale bar, 1 μm.
of the sarcolemma reveal transverse-tubule openings in ordered arrays, and cross fractured necks of the membrane invaginations termed caveolae (Fig. 8). In thin sections cut tangentially to the sarcolemma surface, it becomes apparent that many caveolae share a common neck, and some form complex beaded chains (Fig. 9). Transversely cut sarcolemmata display a conspicuous glycocalyx (Fig. 10) which is continuous with that of the transverse-tubules (Fig. 11). The geometry of the free and junctional components of the sarcoplasmic reticulum (S.R.) (Figs 10, 12) is identical to that observed in myocytes of intact control tissue.

Successful dissociation of myocytes requires that intercalated discs are separated with minimal perturbation. That this requirement is fulfilled is indicated by the well preserved 'half-discs' observed at the ends of isolated myocytes (Fig. 13). The fasciae and maculae adherentes junctions are easily parted without damage owing to removal of their intercellular adhesive constituents during perfusion with the low-calcium collagenase solution. However,
gap junctions do not appear to be separated so readily; thus, the components of both junctional membranes are commonly found associated with one cell. A characteristic feature of isolated myocytes which is seldom apparent in the myocytes of intact tissue is the presence of annular gap junctions in the cytoplasm (Fig. 14).

The contractile apparatus of the rod-shaped isolated myocytes reveals clear I and H-bands indicative of a relaxed state (Figs 6 and 15). The sarcomere length as measured between adjacent Z-bands (Fig. 15) is 1.6 µm. This value is close to that we have previously reported in isolated myocytes examined in thin section [3], and compares with a slack sarcomere length of 1.8 µm determined in fresh or fixed myocytes not processed for electron microscopy [10]. The sarcomere length in myocytes of intact control tissue after perfusion fixation (Fig. 1) is 2 µm, suggesting that some shrinkage of the contractile apparatus occurs during processing for electron microscopy, resulting in a decrease of ~10% in sarcomere length.

Mitochondria in the rod-shaped myocytes display well preserved cristae in a dense matrix (Figs 6, 12 and 15). None of the abnormalities apparent in mitochondria of cells subjected to the calcium paradox [8] or those illustrated here in the rounded, damaged myocytes (Fig. 4), are detected. In favourable sections, the cytoskeletal system of isolated myocytes is visualized (Fig. 9).

**Discussion**

The ultrastructural damage characteristic of the calcium paradox is associated with gross disturbances to myocardial function [13–15]. Although the precise sequence of events leading to the calcium paradox has yet to be fully established, the energy dependent processes of calcium and phosphate ion accumulation by mitochondria, and increased permeability of the sarcolemma to calcium have been clearly implicated [12,8]. Whatever the primary cause of the calcium paradox, the most striking features of the ultrastructural damage that results appear to be related to gross contraction of the myofibrils.

Superficially, the ultrastructural damage observed in the population of rounded isolated myocytes resembles that of the calcium paradox. In both situations gross contraction of the myofibrils could be attributed to a large influx of calcium. If this conclusion is correct, however, the primary causes of such an influx are almost certainly different.

As has been emphasized in the results, to obtain the typical calcium paradox response great care is required to ensure calcium-free conditions. However, for routine isolation of myocytes by the technique of Powell et al. [4], although nominally-free calcium solutions are used, some calcium is present at all stages throughout the isolation procedure [12]. This derives from endogenous calcium, calcium added with the enzyme solution [6], calcium present

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*Figure 10* This field shows a transverse section through the isolated myocyte sarcolemma, revealing details of the surface glycocalyx (GLY). The free sarcoplasmic reticulum (SR) is visible as a tubular network. Scale bar, 0.5 µm
as a contaminant in the collagenase, albumin and salts, as well as calcium leached from the tissue during perfusion. Such calcium levels, although low (~ 10–40 μM), may well be sufficient to saturate high affinity sarcolemmal binding sites, thereby contributing to the maintenance of membrane integrity[1,12,13] in the majority of cells. Those cells that are rounded and damaged in the Powell technique cannot therefore be explained in terms of the calcium paradox.

What, then, is the explanation for the presence of these rounded cells? The ultrastructural observations suggest that mechanical damage, particularly that sustained at the intercalated discs, is the principle factor responsible. Physical separation of individual cells from one another must inevitably involve disruption of the normal intercellular relationships prevailing in intact tissue. The fact that gap junctions are apparently not cleaved apart in an analogous manner to adherentes junctions, makes these a potentially vulnerable site for such mechanical damage.

Our present and earlier[9] observations demonstrating that the majority of isolated myocytes are rod-shaped and well preserved, as judged both by thin section and freeze-fracture electron microscopy, indicate that the cells do not inevitably sustain irreversible mechanical damage. In view of the

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**Figure 15** Isolated myocyte showing general view of the contractile apparatus with clear (Z), (A), (I) and (H) bands, reflecting the degree of relaxation. No evidence of myofilament damage is apparent. Scale bar, 1 μm.

**Figure 11 (Top)** Transverse tubule and junctional sarcoplasmic reticulum organisation in an isolated myocyte. A t-tubule (T) can be seen penetrating into the cell in longitudinal profile, with continuous glycocalyx (G) covering. Transverse profiles of t-tubule/sarcoplasmic reticulum junctions are seen in line with the Z-bands forming 'diads' or 'triads'. Scale bar, 0.5 μm.

**Figure 12 (Middle, left)** High magnification view of junctional sarcoplasmic reticulum (JSR) in an isolated myocyte. Regularly spaced electron-dense processes project from the JSR towards the t-tubule (arrows). An internal electron-dense line is also apparent in the lumen of the junctional SR cistern. Scale bar, 0.1 μm.

**Figure 13 (Right)** A typical image of the intercalated disc region of rod-shaped isolated myocytes. The cytoplasm adjacent to the intercalated disc sarcolemma is well-preserved, with no evidence of disruption of oedema. The normal convoluted geometry of the disc membrane is retained and the fasciae adherentes junctions have been separated in half. Scale bar, 1 μm.

**Figure 14 (Bottom, left)** Isolated myocyte, intercalated disc region. Detail of an annular gap-junction in the cytoplasm. Scale bar, 0.1 μm.
abundance of gap junctions at each intercalated disc[41], it might be predicted that all cells would become damaged if disruption at these junctions was always irreversible. Ultrastructural evidence (unpublished observations) suggests that mechanisms may exist for the repair of minor sarcolemmal damage. It seems, therefore, that it is only when a threshold of damage is exceeded that irreversible disruption occurs and rounded cells result.

Myocytes in isolated hearts subjected to a calcium-free perfusion lose their calcium tolerance when re-exposed to physiological levels of calcium. By contrast, rod shaped myocytes are calcium tolerant, by definition, since they maintain structural and functional integrity in the presence of physiological concentrations of extracellular calcium. Whatever mechanisms underlie the sequence of events involved in the calcium paradox, our detailed ultrastructural observations clearly demonstrate that it is not an indispensable condition that the isolation of cardiac myocytes necessarily leads to this phenomenon.

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References