Contracture in Isolated Adult Rat Heart Cells
Role of Ca\textsuperscript{2+}, ATP, and Compartmentation

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SUMMARY Isolated intact quiescent myocytes from the adult rat were used as a model system for investigating the determinants of contracture induced by metabolic deprivation. The model simulated the pattern of contracture and ATP decline seen in the intact heart during ischemia. Three new insights into the contracture process were gained: (1) in the quiescent cell system, the rate of onset of contracture was independent of external Ca\textsuperscript{2+}, supporting the view that the Ca\textsuperscript{2+} dependence of the rate of onset in the whole heart is related to beat-dependent substrate utilization; (2) the second phase of ATP decline was paralleled by a decline in the percentage of cells which had not undergone contracture, suggesting that—in any cell—contracture is immediately preceded by a total loss of ATP; and (3) oligomycin delayed the onset of contracture by 55 ± 12%, suggesting that mitochondrial ATPase activity is a significant drain on energy resources in the quiescent ischemic heart.


AN important step in the analysis and understanding of any property of the heart is to define and characterize the equivalent property at the level of the myocardial cell. The behavior of the whole organ can then be understood in terms of the intrinsic cellular property modulated by extrinsic factors such as supracellular organization and communication and vascular control of blood flow. One way of determining the contribution of such extrinsic factors is to attempt to reconstitute or mimic the behavior of the whole organ, with respect to some particular property, using a suspension of isolated myocytes. Until recently such studies were not realistic, because of the lack of a preparation of isolated adult myocytes which are truly intact. Methods have been available for 20 years for the preparation of myocyte suspensions which show the characteristic rod-shaped morphology of cells in vivo; however, these preparations undergo contracture when exposed to physiological levels of Ca\textsuperscript{2+}, suggesting that the integrity of the cell membrane is in some way compromised. However, a number of procedures have been recently reported for preparing myocytes which remain rod-shaped in the presence of Ca\textsuperscript{2+}, suggesting that the integrity of the cell membrane in some way compromised. However, a number of procedures have been recently reported for preparing myocytes which remain rod-shaped in the presence of Ca\textsuperscript{2+} (Powell and Twist, 1976; Kao et al., 1980; Haworth et al., 1980). The latter preparation forms the basis of this study of contracture.

Ischemic contracture of the heart is an uncommon complication of normothermic open heart surgery (Cooley et al., 1972). The analogy to the rigor state of skeletal muscle was quickly recognized (Katz et al., 1972), and in support of this concept, the loss of tissue ATP has been shown by Hearse et al. (1977) to precede and accompany the contracture process in the rat heart. By comparing the decline of tissue ATP with the onset of contracture as measured by the pressure in a ventricular balloon, these authors concluded that contracture was initiated when the cellular ATP content decreased to approximately 12 µmol/g dry weight. A figure of 16 µmol ATP/g dry weight was reported by Jarrahan et al. (1978) for the onset of contracture in the adult rabbit heart. A limitation of these studies is that both the contracture measurement and the ATP measurement reflect averages over the whole myocardium, and do not give information on a possible heterogeneous response of myocardial cells to the ischemic insult. Although it is difficult to mimic precisely the condition of ischemia in the whole heart by using a preparation of isolated cells, a particular advantage of using isolated myocytes as a model system is that the extent of the heterogeneity of the contracture response of the cells can be quantitated readily and correlated with average ATP levels. The importance of this advantage is demonstrated in the study reported here.

Methods

Isolation of Ca\textsuperscript{2+}-Resistant Myocytes

Cell suspensions were prepared as previously described (Haworth et al., 1980). Two female retired breeder Sprague-Dawley rats were anesthetized with intraperitoneal injection of 0.4 ml 5% thiamylal sodium (Surital) and the hearts excised. After retrograde (Langendorff) perfusion at 37°C for 5-10 minutes with a Krebs-Henseleit bicarbonate medium, first with Ca\textsuperscript{2+} and then without Ca\textsuperscript{2+}, the hearts were digested for 40 minutes by recirculating...
perfusion with medium containing collagenase (0.55 mg/ml) and hyaluronidase (0.33 mg/ml), and no added Ca²⁺. The hearts were cut up and digested in a shaking incubator for a further 90 minutes at 37°C with the perfusion medium plus 1 mM CaCl₂ and trypsin (0.01 mg/ml). This stage of trypsin digestion was shown to convey resistance to Ca²⁺ (Haworth et al., 1980). Cells were filtered, washed, and used immediately.

**Protein Measurement**

The biuret procedure was used to measure protein.

**Aerobic Incubation Conditions**

Cells were washed and suspended at room temperature in a medium containing: 118 mM NaCl, 4.8 mM KCl, 25 mM 4-morpholinopropane sulfonate (MOPS), 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1 mM CaCl₂, and 11 mM glucose. The pH had been adjusted to 7.0 with NaOH. The suspension was incubated at 37°C in a Dubnoff metabolic shaking incubator, set at 80-90 cycles/min, and allowed to equilibrate with air. The protein concentration was measured at this time and adjusted to 2.4 mg/ml. Experiments were initiated typically after 30 minutes of incubation, by the division of the suspension as appropriate and the addition at "time zero" of reagents indicated in the figures. Under these conditions there was no measurable decline of ATP content or percent rod-shaped cells in the control for at least 70 minutes; adjusting the pH to 7.4 made no difference to the stability of the cells.

**Anaerobic Incubation Conditions**

Cells were washed and suspended at room temperature in a medium containing: 118 mM NaCl, 4.8 mM KCl, 25 mM MOPS, 1.2 mM KH₂PO₄, 11 mM glucose, 3 mM pyruvate, 3 mM malate, and 1 mM EDTA, pH 7.0. The suspension (2.5 mg/ml) was cooled to 0°C, because contracture was inhibited at 0°C, and digitonin (1 mg/ml ethanol) added to a final concentration of 25 μg/ml suspension. Three drops were removed and checked to ensure that trypan blue could permeate all cells (see above). The cells, now known to be permeable, were washed three times in about 10 volumes of the above buffer at 0°C, and finally resuspended in buffer like the above except for 1.2 mM MgCl₂ and 0.1 mM EGTA in place of the EDTA. That this procedure was successful in depleting the myofibrils of ATP was shown by the following observation: cells warmed up to 37°C did not undergo contracture unless a low level of ATP (e.g., 20 μM) was added to the medium.

**ATP and Creatine Phosphate Measurements**

One milliliter of ice cold 16% perchloric acid was added to the tube containing the cells, immediately after the three drops were removed for fixation. Extracts were neutralized with KHCO₃ and frozen at -70°C. Thawed samples were centrifuged, and ATP levels measured directly by high pressure liquid chromatography (HPLC), using a Perkin Elmer Series 2 Liquid Chromatograph fitted with a Whatman Partisil PX5 10/25 SAX pre-packed column. Eighty microliters of each sample were applied, and eluted in 20 minutes by a linear salt gradient ranging from 1.2 mM KH₂PO₄ adjusted to pH 3.5 with acetic acid, to 0.6 mM KH₂PO₄, adjusted to pH 3.5 with acetic acid. The flow rate was 1 ml/minute. Effluent sample and standard nucleotides were detected at a wavelength of 254 nm. Creatine phosphate was measured by the method of Lowry and Passonneau (1972), except that a 50 mM Tris MOPS buffer, pH
Glycogen Measurements

Cell glycogen was extracted with acid by the method of Lowry and Passonneau (1972) and digested with α-amylase by the method of Hearse and Chain (1972); the release of glucose was measured by the method of Lowry and Passonneau (1972).

To a 1-ml sample of cell suspension were added 50 μl of 1 N HCl, and the tube was placed in a boiling water bath for 10 minutes. When cool, 0.4
ml 1 M sodium acetate (pH 5.0) was added, followed by either 0.1 ml 0.5 mg/ml amyloglucosidase (E.C. 3.2.1.3) or 0.1 ml buffer blank. The tubes were incubated 1 hour at 37°C. To measure the glucose released, the suspensions were neutralized by the addition of 1.5 ml solution containing 267 mM Tris base and 2 mM MgCl₂, followed by the addition of 0.5 mM ATP, 0.5 mM NADP⁺, 0.2 U glucose-6-phosphate dehydrogenase, and 2 μg/ml hexokinase. After 10 minutes, the tubes were centrifuged, and the extent of NADP⁺ reduction was measured from the absorbance of the supernatant at 340 nm. The validity of the assay was established by several criteria. Standards of liver glycogen showed precise linearity and complete recovery within a few percent. That all the amylose-releasable glucose came from glycogen was confirmed by parallel measurements with the phosphorylase α assay of Lowry and Passonneau (1972): both before and after anaerobic incubation, the amylose-digestible carbohydrate was 40% digestible by phosphorylase α. The liver glycogen standard was found to be 30% digestible by phosphorylase α. The amylase assay was used in preference to the phosphorylase α assay of Lowry and Passonneau (1972) for this very reason: the preparation of phosphorylase α (obtained from Sigma Chemical Co.) contained insufficient debranching enzyme to allow the complete degradation of the glycogen.

Lactic Dehydrogenase Release

After removal of three drops for fixation, the remaining cells were spun down using a Beckman Microfuge B bench centrifuge. Lactic dehydrogenase activity in the supernatant was measured as follows: to 3 ml “aerobic incubation” buffer without CaCl₂ or glucose, containing 1 mM NAD⁺ and 5 mM lactate, was added 7.5 to 30 μl supernatant. The initial rate of NAD⁺ reduction was measured from the absorbance increase at 340 nm. A figure for 100% release of lactic dehydrogenase activity was obtained by first treating the cells with 25 μg/ml digitonin.

Source of Special Reagents

The uncoupler carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Pierce Chemical Company. Bovine serum albumin (BSA), fatty-acid free fraction V, was obtained from Miles Laboratories, Inc.

Reproducibility

The experiments shown in each figure were repeated at least once to ensure reproducibility. Data from different experiments were not generally combined, however, because each experiment was internally controlled.

Results

Cell Morphology

Cell suspensions typically contain a mixture of 60-70% rod-shaped cells (Fig. 1A) and 30-40% of cells that have rounded up (Haworth et al., 1980). When a suspension is subjected to the anaerobic conditions described in Methods, the rod-shaped cells in time undergo a process of contracture to a form where the sarcomere period is still readily discerned, but is shortened dramatically (Figs. 1B and 2). Note that the designation “rod-shaped” applies specifically to relaxed cells with the long sarcomere length, regardless of shape. All cells become shorter when contracture occurs; however, shape is not an infallible guide, since cells initially show a wide distribution of shapes and sizes. Sarcomere length is the ideal measure. For any individual cell, there is a lag period after the initiation of anaerobiosis during which the cellular morphology as seen in the light microscope is unaltered. Contracture, when it occurs, is very sudden, as evi-
denced by the fact that aliquots of cell suspension fixed at intermediate times show a mixture of cell types (Fig. 2): relaxed rod-shaped cells of unaltered morphology (Fig. 1A), contracted cells (Fig. 1B), and round cells with no discernible sarcomere bands. Very few cells were seen in an intermediate configuration between relaxed and contracted; cells in this category were counted as contracted. The number of rod-shaped and non-rod-shaped cells could therefore readily be quantitated at any time. Figure 3 shows the time course of onset of contracture in the heart cell suspension in a similar experiment. The curve displays a lag, followed by a rapid drop, and then tails off. Figure 3 also shows that the rate of onset of contracture in isolated quiescent myocytes is unchanged when Ca²⁺ is removed with EGTA.

ATP Levels

The correlation between contracture and the loss of ATP is shown in Figure 4. An initial drop in ATP levels occurred before contracture began. ATP levels appeared to stabilize somewhat, before undergoing a second drop temporally associated with contracture.

To us, a disturbing feature of Figure 4 was the significant level of ATP remaining when contracture was complete. On investigating this residual ATP in the cell suspension, we found that its level was critically dependent on the brief shake given the tubes to resuspend the cells prior to removal of drops for fixation and morphological examination. This suggested to us that the level of ATP in the contracted cells could well have been near zero, but it was given an artifactual boost by oxidative phosphorylation from residual oxygen in the buffer, when the tube was shaken. Therefore, the experiment was repeated with oligomycin added just prior to the 37°C incubation, in order to completely inhibit oxidative phosphorylation. The results are shown in Figure 5. The ATP drop is once again biphasic. However, the ATP axis has been scaled to show that the second phase of the ATP drop is now precisely (within experimental measurement) coincident with the onset of contracture.

Permeability Changes

Figure 6 shows that anaerobic incubation induces a non-specific permeability change in the sarcolemma, as measured by trypan blue uptake or by enzyme release, but the event occurs long after contracture.

Substrate Levels and Contracture

Figure 7 shows an experiment where glycogen levels and extent of contracture both were measured. Two things are noteworthy. First, there is a rapid rate of loss of glycogen, which gradually levels off to zero rate of loss. Second, the glycogen level of the suspension did not decrease to zero when all cells were contracted, but a baseline level of 24 nmol glucose equivalents/mg was left. We have,
Contracture was found to be inhibited by the provision of glucose (Fig. 8). A shaking incubator was used to ensure adequate access of glucose to the cells, and mitochondrial respiration was inhibited by the addition of rotenone. These conditions also prevent any inhibition of glycolysis by acidosis, since the medium was well buffered. No decline in the pH of the medium was measurably over the course of the experiment. It is clear that while cells without glucose underwent contracture with a time course similar to that of cells in the anaerobic model, the provision of glucose dramatically delayed the onset of contracture by at least 2 hours.

**Effect of Oligomycin**

Figure 8 also demonstrates a rather striking effect of oligomycin on the rate of onset of contracture. This can also be seen in the anaerobic model by comparing Figures 3 and 4 (no oligomycin) with Figures 5 and 7 (with oligomycin). Time to 50% contracture in the anaerobic model was 23.2 ± 3.1 min without oligomycin, and 35.8 ± 5.2 min in the presence of oligomycin (n = 6 experiments, \( P < 0.001 \)), which represents a delay of contracture of 54.7 ± 11.6%. A much more dramatic effect of oligomycin was observed on contracture induced by the addition of the uncoupler FCCP under aerobic incubation conditions (Fig. 9).

**Effect of Bovine Serum Albumin**

A small delay in the onset of contracture was observed when bovine serum albumin (BSA) was included in the anaerobic incubation medium (Fig. 10). The increase in time to half contracture (t_{1/2})...
Figure 7  Contracture and the loss of glycogen in the anaerobic model. O, onset of contracture; ●, loss of glycogen. No CaCl₂ was added in this experiment. Protein concentration was 2.85 mg/ml.

Figure 8  Effect of glucose and oligomycin on contracture of rotenone-inhibited cells in suspension. O, no addition; ●, 10 μM oligomycin; △, 11 mM glucose; ●, 11 mM glucose plus 10 μM oligomycin. All suspensions contained 2.5 μM rotenone. Protein concentration was 1.65 mg/ml. Incubation conditions were as described in Methods for aerobic incubations, except that glucose was included only where shown.

was 7.9 ± 4.9 min (mean ± sd, five experiments). The increase in t₁/₂ produced by oligomycin was 10.8 ± 4.4 min, and the increase produced by oligomycin plus BSA was 18.6 ± 3.3 min. Thus, the benefits of BSA and oligomycin appear to be additive.

Discussion

The incubation conditions described here as "anaerobic" included not only oxygen starvation, but also substrate starvation and stagnation of the cell suspension, which resulted in a degree of acidosis. These conditions could therefore be closer to those produced by global ischemia of the whole heart. This possibility is supported by the striking similarity between the changes observed in our model system and those observed by Hearse et al. (1977) in the whole rat heart subject to global ischemia: the sigmoidal onset of contracture (Figs. 1 and 3), the biphasic drop of ATP levels (Fig. 4), and the sharp decline in glycogen content (Fig. 7). This is a demonstration of the extent to which the whole heart behaves as the simple sum of its component myocardial cells. The major difference is the slower rate of onset of contracture in the cell model. The time for 50% of cells to contract was 23.1 ± 3.9 min (n = 6), compared with about 12 minutes for time to 50% contracture in the intact rat heart (Hearse et al., 1977). Part of this difference is probably due to the fact that the isolated myocytes are quiescent. Hearse et al. (1977) found that cardioplegia with 16 mM KCl, or with the Ca²⁺-blocking agent D600, or with no Ca²⁺ in the pre-perfusate, resulted in an extension of the time for 50% contracture to up to 25 minutes. A similar inhibition of contracture has been observed with nifedipine, another Ca²⁺ antagonist, by Henry et al. (1977). The delay of contracture...
Inhibition by oligomycin of contractive induced by uncoupler in cells in aerobic suspension. O, no addition; •, 0.5 μM FCCP; △, 33 μM oligomycin; ▲, 33 μM oligomycin plus 0.5 μM FCCP. Protein concentration was 2.4 mg/ml.

The precise parallel found between contracture and the second phase of ATP decline implies that once a cell has established the new steady state level of ATP after the initiation of anaerobic incubation it continues to maintain this steady state level right up to some point in time when the level suddenly drops to zero and contracture occurs. Intermediate levels of ATP measured during the second phase of ATP decline are thus the average of a fraction of rod-shaped cells which are continuing to maintain their ATP levels and a fraction of contracted cells which contain no ATP. This conclusion makes the lack of effect of Ca^{2+} on the onset of contracture more understandable. The initial ATP drop must be insufficient to affect Ca^{2+} homeostasis. The second ATP drop could well allow Ca^{2+} to enter. However, this event is so close in time to contracture that Ca^{2+} appears not to affect its rate of onset. The strength of contracture, of which we have no measure here, could on the other hand be quite enhanced by the presence of Ca^{2+}.

How low must the cellular ATP level drop before contracture occurs? It is known from studies with isolated myofibrils that low levels of ATP-Mg^{2+} stimulate ATPase activity and trigger syneresis (Weber et al., 1969), a phenomenon presumed to be related to contracture. This effect of low levels of ATP-Mg^{2+} has been interpreted in terms of the contractile process being turned on in the absence of Ca^{2+} when a critical level of actin-myosin rigor complexes forms (Bremel and Weber, 1972). The rigor complexes form when levels of ATP-Mg^{2+} become insufficient to saturate the myosin binding sites. The level of ATP-Mg^{2+} needed to inhibit this Ca^{2+}-independent process in isolated skeletal muscle myofibrils is about 100 μM (Weber et al., 1969). We have made a similar observation on isolated...
heart cells rendered permeable with digitonin and depleted of endogenous ATP. When such a suspension of cells (see Methods for details of preparation) was incubated in the presence of 100 µM ATP-Mg²⁺ at 37°C, the cells were still relaxed after 2 minutes. Cells exposed to <50 µM ATP-Mg²⁺, on the other hand, underwent immediate contracture. How does this compare with the level of ATP measured in the anaerobic cell? The value of 15 nmol ATP/mg protein is shown in Figure 5 to correspond to a population of 55% cells relaxed (rod-shaped). This corresponds to about 27 nmol ATP/mg rod-shaped cell protein, which still corresponds to a cellular level of ATP of about 5 mM, using a cell density of 5 µl/mg protein. It is therefore apparent, from even a rough calculation, that the ATP loss must be a sudden drop of at least 20-fold.

What causes the sudden loss of ATP? It cannot be explained in terms of a gross change in membrane permeability, since that occurs long after the onset of contracture (Fig. 6). Could it be explained in terms of a sudden arrest of glycolysis? It was noted by MacGregor et al. (1975) that the onset of ischemic contracture in the canine left ventricle was preceded by an inhibition of anaerobic glycolysis. Hearse et al. (1977) showed that glycogen stores in the rat heart had dropped to less than 4% of their initial value by the time ischemic contracture was completed. The suddenness of the drop in ATP levels in the anaerobic cells (Fig. 5), and the similar time course of onset of contracture in a buffered medium (Fig. 8), both argue against a gradual inhibition of glycolysis by acidosis. On the other hand, the presence of residual glycogen at the end of contracture argues against exhaustion of cell glycogen as being the event that triggers the sudden decline in ATP. We have therefore sought to determine the location of this residual glycogen. Three lines of evidence support the view that the residual glycogen was located in the dead (trypan blue permeable) cells at time zero, and it was never used up during anaerobic incubation. First, we have found that cells with ruptured sarcosomes are still able to retain substantial amounts of glycogen. Treatment of good cells with digitonin (see Methods), followed by washing as during preparation of the cell suspension, resulted in the removal of only 40-50% of the cell glycogen. This glycogen was not used up when the digitonin-treated cells were exposed to 60-minute anaerobic conditions. Second, we have found that release of the residual glycogen from cells subjected to anaerobic conditions cannot be stimulated by digitonin, showing that the release of this glycogen is not limited by sarcosomal integrity. Third, we have confirmed the observation of Hearse et al. (1977) that 60 minutes of global ischemia of the whole heart, in which all the cells are initially intact, results in almost total depletion of the glycogen. This evidence, therefore, supports the conclusion that glycogen in the rod-shaped cells is completely depleted by the anaerobic incubation, causing the sudden loss of ATP and subsequent contracture.

If the above interpretation is correct, the observation that some cells last much longer than others could mean that some cells contain much higher levels of substrate stores than others. The difference in time to contracture could not be caused by a non-uniform environment in the stagnant suspension, because a similar phenomenon was seen when the cell suspension was shaken (Fig. 8).

Since oligomycin is a powerful and specific inhibitor of mitochondrial ATP synthesis, it at first appears paradoxical that it should prolong the survival time of cells in such an energy-starved state. Oligomycin does, however, also specifically inhibit mitochondrial ATP hydrolysis, and this must be the cause of its protective effect shown in Figure 8, since mitochondrial ATP synthesis had already been blocked by rotenone or, in the case of Figures 3, 4, 5, and 7, by anoxia. Such a conclusion is borne out by the ability of oligomycin to protect cells against the rapid onset of contracture induced by the addition of the uncoupler FCCP, which is known to induce an active mitochondrial ATPase activity as well as uncoupling oxidation from phosphorylation (Fig. 9). This raises the question of whether the mitochondrial ATPase inferred to be present during anoxia results from the presence of endogenous uncouplers, such as free fatty acids. Figure 10 shows that, although a slight beneficial effect of BSA is apparent, it is smaller than the oligomycin effect, and moreover the benefits of BSA and oligomycin appear to be additive. We must, therefore, conclude that either added BSA is unable to bind the free fatty acid causing the ATPase, or else the ATPase is not caused by free fatty acid.
(1980) found that all cells in a buffered medium exposed to anoxia were contracted after an hour, even in the absence of Ca²⁺, and whether glucose was present or not.

It has always been recognized that measured tissue levels of metabolites do not necessarily reflect the situation in every cell of the tissue. The present study demonstrates, however, that this factor can be extreme: temporal variations in average metabolite levels can be an absolute reflection of cellular heterogeneity.

Finally, the behavior of ATP levels within individual cells demonstrated here for adult myocytes may well be a property of cells of any tissue exposed to anoxic or ischemic conditions. It is true that contracture in myocytes will produce an ATPase activity that will not be present in non-muscle cells. On the other hand, we have given evidence that the ATP drop must occur before the contracture is induced. If we are correct in pinpointing deficiency in substrate as the origin of the sudden drop, there is no reason to suppose that this phenomenon should be unique to the heart. The uniqueness of myocytes may lie simply in their possessing an inbuilt ATP monitor, namely, the myofibril, which has allowed this ATP catastrophe to be detected.

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