Direct Observation of the "Oxygen Paradox" in Single Rat Ventricular Myocytes

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SUMMARY. By phase contrast microscopy with video length tracking, we followed the sequence of morphological changes in individual isolated rat ventricular myocytes during anoxia followed by reoxygenation. Cells appeared normal during early anoxia. After a duration of anoxia $T_1$, which varied from 17-47 minutes in different cells, each cell abruptly contracted an average of 33% in length to an inert rectangular form presumed to be a rigor state. Cells which were reoxygenated before the onset of rigor showed normal morphology and an unchanged extent of shortening on field stimulation, compared to control. Cells that were reoxygenated after a time in the rigor state, $T_2$, either partially recovered to a shortened rectangular form capable of stimulated twitches or rounded up rapidly to a disordered hypercontracture form. The distribution of $T_1$ was the same for cells which recovered and which hypercontracted. In contrast, the outcome of reoxygenation depended markedly on $T_2$: all cells that were reoxygenated after less than 10 minutes of rigor recovered function, whereas all cells that spent more than 20 minutes in rigor hypercontracted when reoxygenated. The hypercontracture appears to be the cellular analog of the "oxygen paradox" in whole hearts. Its occurrence is reliably related to duration of rigor state but not to duration of hypoxia, because of marked cellular variability in the time of onset of rigor.

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REFLOW of myocardium that has been ischemic for a prolonged period produces a characteristic histological picture of "contraction band necrosis" (Kloner et al., 1974). A similar phenomenon, the "oxygen paradox," is produced when an isolated heart that has been perfused anoxically and without glucose is reoxygenated (Hearse et al., 1975). The introduction of oxygen is associated with abrupt development of a patchy contracture state, with disruption of sarcoclemmae and intercalated discs, contraction bands and Ca"" deposition in the mitochondria. On the basis of the timing of these events, it has been proposed (Ganote and Kaltenbach, 1979) that the primary event is a violent contracture, which produces cellular disruption, Ca"" loading, and enzyme release by a mechanical disruption of the intercellular attachments. Because isolated cardiac myocytes have no such attachments, the analog, if any, of the oxygen paradox in these cells would be of great interest.

Previous studies of hypoxia and reoxygenation in isolated cells have been performed in suspension or layers, sampled at discrete times (Acosta and Puckett, 1977; Acosta and Li, 1980; Altschuld et al., 1981; Cheung et al., 1982; Murphy et al., 1982; Hohl et al., 1982; Piper et al., 1984). The behavior of isolated myocytes under hypoxia is heterogeneous. Haworth et al. (1981) noted that the sarcomere lengths of hypoxic myocytes in suspension are bimodally distributed. As hypoxia is prolonged, there is an increase in the fraction of "short" cells and a decrease of "long" cells; the adenosine triphosphate (ATP) content of the suspension is proportional to the number of long cells. They proposed that an individual cell regulates its ATP level by means of anaerobic glycolysis until its energy reserves are exhausted; it then abruptly becomes ATP depleted and shortens to a rigor form. If this interpretation is correct, the averaging of cells in suspensions or dense layers might be quite misleading. We therefore undertook to observe, by direct phase contrast microscopy, the "histories" of individual myocytes during hypoxia and reoxygenation.

Methods

Isolated rat ventricular cells were prepared as previously described (Silver et al., 1983). Briefly, hearts from 6- to 7-month-old Wistar rats were perfused for 15 minutes with Krebs-Ringer bicarbonate buffer containing 0.1% collagenase type II (Worthington), 50 μM Ca"", 10 mM glucose, at pH 7.4 and 37°C. The left ventricle was dissected free, minced, and the fragments pipetted gently and repeatedly. The supernatant extract was filtered through gauze and allowed to form a pellet by gravity. We washed the cells repeatedly with Spinner's medium (Gibco), progressively increasing concentrations of Ca"", and resuspending them, finally, in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 1.8 mM Ca"". This resulted in a cell suspension containing 60-75% rod-shaped Ca""-tolerant myocytes. These were plated into 25-mm glass Petri dishes containing glass coverslips and...
FIGURE 1. Apparatus used to produce hypoxia in cells. Cells were in glass Petri dishes flushed by ultra-pure argon gas. The medium was exchanged for hypoxic medium (flask) by pinching the rubber segment of the gas line to raise pressure up in the flask, avoiding valves. During the hypoxic period, the liquid phase was left stationary, since the liquid is 30 times more susceptible to oxygen contamination than the gas phase because of relative solubilities.

were incubated for 1 hour. The medium with unattached cells was then washed off and replaced with HEPES buffer containing (in mM): NaCl 137, KCl 5, MgSO<sub>4</sub>·7 H<sub>2</sub>O 1.2, HEPES 20, glucose 15, CaCl<sub>2</sub> 1.5. Virtually all cells that remained attached to the coverslip were rod shaped, with regular sarcomere patterns, and contracted synchronously in response to field stimulation.

Attached cells were made hypoxic in glass Petri dishes (Fig. 1). The medium in the Petri dish was exchanged 10 times with glucose-free HEPES buffer with 1.5 mM Ca<sup>2+</sup> that had been equilibrated for 2 hours with ultra-pure argon gas (99.9995% Matheson). The upper part of the Petri dish was enclosed in a Lucite chamber flushed under positive pressure with argon pre-humidified by equilibration with the buffer flask. Gas was delivered through copper and glass tubing except for two 1-inch segments of low-oxygen permeability Fluran rubber to provide flexibility. The medium was exchanged through stainless steel tubing by partially occluding one of the rubber segments in the gas line to alter the pressure relationship between the flask and the chamber, avoiding valves. Measurements with a Clark oxygen electrode did not detect any oxygen in the gas or liquid phases; the dissolved P<sub>O</sub><sub>2</sub> was estimated to be less than 0.5 torr at most. The system was maintained at 37°C for all experiments. The cells were viewed by phase contrast microscopy using a Leitz Diavert inverted microscope; the image was projected onto a Panasonic television camera, and the video signal was recorded on a Panasonic VHS cassette recorder. The cell length was monitored by a video edge tracking system (Instrumentation for Physiology and Medicine, San Diego) from the recorded image. Cells were reoxygenated by flushing the Lucite chamber with air. Cells were field stimulated before and after hypoxia by a Grass stimulator via platinum-iridium wires immersed at opposite sides of the Petri dish; stimulation was avoided during hypoxia to prevent electrolytic production of oxygen.

### Results

When the above precautions were taken to ensure adequate hypoxia, cells were observed to undergo a highly reproducible sequence of events (Fig. 2). Initially, there was no alteration of light microscopic morphology or cell length. After a period of hypoxia which varied from cell to cell, ranging from 17–47 minutes, each cell underwent a smooth longitudinal collapse (Fig. 3A) which occurred abruptly, i.e., over about 10 seconds, to become an "R form," a short rectangular form with a faintly visible sarcomere pattern and an enhanced contrast of the myofibrillar structure. The cell length decreased by 32.5 ± 7.9% (mean ± SD, n = 29) on entering the R-form. Cells in the R-state remained perfectly inert, without motion or change of morphology, as long as hypoxia was maintained. We presume that the R-form is a rigor state, though this cannot be proven in the absence of ATP measurements on single cells. The initial period of hypoxia, measured from the completion of the last exchange of medium to the onset of the R-state, was defined as T<sub>1</sub>, and varied in each cell and was unpredictable.

After onset of the R-state, hypoxia was continued for an additional time T<sub>2</sub> at which time air was blown into the space above the Petri dish. One
minute after reoxygenation, all cells underwent a "recontraction," a brief longitudinal shortening, followed by a partial relaxation (Fig. 3A). The length of the cell at this point was close to that in the R-state or somewhat shorter.

Beyond this point, there were two possible outcomes. Some cells (17/29) underwent partial recovery. These cells, after a latent period of up to several minutes, began to display spontaneous wave-type contractions (Capogrossi and Lakatta, 1985). These often reached a frequency of one every 3-5 seconds before slowing down to a rate of 1-4/min, or ceasing altogether. Partially recovered cells remained rectangular and held constant lengths or elongated slowly, although never to control length. They reached a steady state which remained unchanged for at least an hour after reoxygenation. Most (68%) partially recovered cells produced a synchronous twitch contraction in response to field stimulation (Fig. 3B). Since the cells were short, and often located in an area far from the stimulating electrodes, it is possible that those which could not be field stimulated were nevertheless electrically intact.

The second possible outcome of reoxygenation was hypercontracture. These cells (12/29) exhibited rapid asynchronous oscillations (up to 4 Hz) and progressive contracture immediately following reoxygenation. Within 2 minutes, they had entirely rounded up into blebbed forms with absent sarcomere pattern and disordered myofibrils, shorter along the longitudinal axis than transversely. They were unresponsive to electrical stimulation and resembled in all respects the hypercontracted cells produced by massive Ca²⁺ overload.

To determine the influence of the durations of the phases of hypoxia $T_1$ and $T_2$ on the occurrence of hypercontracture, we plotted the cumulative distributions of $T_1$ and $T_2$ with cells grouped retrospectively according to their outcome. The cumulative distribution of $T_1$ (Fig. 4A) is a "survival curve" with "death" defined as entry to the R-state. The times $T_1$ were distributed in the same way for cells which recovered and for those which hypercontracted; the mean $T_1$ was 32.7 ± 1.9 minutes (mean ± sem) and 29.9 ± 2.3 minutes for the two groups, respectively ($P = NS$). The duration of hypoxia before development of rigor was thus not a determinant of the fate of the cell when reoxygenated. In contrast, the distributions of $T_2$, the duration of the rigor state before reoxygenation, differed markedly according to the outcome (Fig. 4B). The distribution of $T_2$ for the entire population was under the control of the ex-
theory of the membrane damage which occurs upon reoxygenation of intact tissue. More recently, Piper et al. (1984) reported studies of myocytes attached to plastic Petri dishes in a nitrogen atmosphere. They described a progressive decrease of sarcomere length which correlated with a progressive fall in dish ATP levels. On reoxygenation, there was complete ultrastructural recovery. Only after an hour of anoxia were any rounded cells seen; after 2 hours, only 30% of cells were rounded, and there was no change in the number of rounded cells upon reoxygenation.

Our results show that anoxic and reoxygenation injury in cardiac myocytes is a "quantized" phenomenon, with each cell following its own timetable. This is in agreement with the inferences of Haworth et al. (1981) and Altschuld et al. (1981). The startling abruptness of the transition to the R-form may reflect the regenerative nature of the process: once myofilaments are activated by the lowering of ATP levels (generally thought to occur below 50 μM), they will rapidly consume the remaining ATP. We have confirmed the existence of a rapid hypercontracture in response to reoxygenation. In addition, we have shown for the first time that some cells which have entered a rigor state can partially recover function, including the ability to twitch in response to electrical stimulation, which implies relative intactness of sarclemal ionic permeabilities and energy production capability. While these cells remain markedly shortened and are clearly damaged, the outlook for their possible salvage is better than for the "viable rounded" cells or nonviable leaky cells which have been described previously.

The apparently inert R-form cell undergoes a time-dependent process which makes it vulnerable to hypercontracture at the time of reoxygenation. The nature of this process is conjectural. Although the hypercontracture itself resembles a Ca ++ overload state, the nature of the damage when the rigor state is prolonged is probably complex, with sodium overload, failure of membrane repair, and oxidative radical damage each possibly contributing. It is noteworthy that in five preliminary experiments we have found that reducing extracellular calcium to 50 μM prevented the abrupt hypercontracture in cells with T2 = 30 minutes, suggesting that calcium is important in the process.

It seems logical to identify the hypercontracture event with the oxygen paradox in whole hearts with the understanding that the damage produced is probably amplified by mechanical disruptions. However, the "recontraction" which occurs in all R-form cells at the time of reoxygenation might itself be sufficiently strong to produce mechanical disruption of intercellular attachments in intact hearts. Therefore, in using the isolated cell model to search for interventions to protect the myocardium against reflow injury, the effect of such interventions on all stages of the sequence shown in Figure 1 needs to be considered.

Our results differ from those of Piper et al. (1984),
with an apparently similar preparation, even after allowing for the effects of averaging over cellular inhomogeneity. They report no increase in rounded forms after reoxygenation. In addition, they observed continual rounding of cells during the second hour of hypoxia, at a time when all of our cells have entered the inert R-form. Because of the small number of cells which can be studied by continuous individual observation, we may have missed a "tail" of cells with long values of TV. However, the 95% confidence limit for the fraction of cells in such a tail is only 10%, and the probability that the tail could contain the 30% of cells which were reported to round up during the second hour is less than 0.0001, given that we observed none. It seems likely, therefore, that the phenomena in their study differed from ours. The reasons for this difference are unclear, and might include difference in the age of the rats (ours were 3 months older) or their state of nutrition or glycogen stores. It is also possible that there was a difference in the degree of hypoxia. In whole tissues or dense cell suspensions, anoxia is achieved by consumption of oxygen by the cells; this mechanism is not available for sparse cells in a Petri dish. The concentration of oxygen at which cardiac myocytes become "anoxic" is not known. In isolated hepatocytes, cytochromes become fully oxygenated at a Po2 of 3 torr (Jones and Kennedy, 1982), so it is possible that significant oxidative phosphorylation can take place even when the Po2 is a fraction of a torr. We were unable to produce our results using plastic (polystyrene) Petri dishes and commercial USP nitrogen (99.5%). It is possible that outgassing of dissolved oxygen from the plastic substrate could influence the metabolic regulation of attached cells even when the supernatant contains insufficient oxygen to be detected with an oxygen electrode.

Further clarification of the biochemical basis of the states indicated in Figure 2 awaits the development of techniques for the measurement of ATP and ions in single cardiac cells under conditions of extreme hypoxia. The morphological sequence observed in this study underlines the importance of accounting for cellular heterogeneity in studies of reoxygenation injury. If the cellular heterogeneity seen in isolated cells exists in whole tissue, then the changes in contraction duration and strength after reoxygenation (Lakatta et al., 1975) may be due entirely to those cells which have passed through the rigor state and sustained permanent damage.

References

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