A model of low-flow ischemia and reperfusion in single, beating adult cardiomyocytes

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Maddaford, Thane G., Cecilia Hurtado, Salisha Sobrattee, Michael P. Czubryt, and Grant N. Pierce. A model of low-flow ischemia and reperfusion in single, beating adult cardiomyocytes. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H788–H798, 1999.—The present study was undertaken to comprehensively characterize low-flow ischemia and reperfusion in single adult cardiomyocytes and to determine whether it is important to control contractile activity. The ischemia-mimetic solution was hypoxic, acidic (pH 6.0), and deficient in glucose but contained elevated KCl. Cardiomyocytes were stimulated to contract throughout ischemia and during reperfusion with control perfusate. After the ischemia-reperfusion insult, cells exhibited poor recovery of active cell shortening, a decrease in passive cell length, increased frequency of necrosis, lower ATP content, and evidence of the generation of oxygen-derived free radicals within the cells. Intracellular lactate concentration increased, pH decreased, and Ca\(^{2+}\) transients were depressed during the ischemic insult, but the latter two parameters recovered partially on reperfusion. Basal intracellular Ca\(^{2+}\) concentration was elevated during ischemia and early into reperfusion. Recovery was attenuated in cells that were electrically stimulated to contract throughout ischemia. The duration of ischemia, stimulation frequency, and composition of the ischemia-mimetic solution were important variables. The inclusion of 10 mM lactate in the ischemia-mimetic solution significantly aggravated all the parameters examined above. Our data demonstrate that 1) an ischemia-mimetic solution administered to single, isolated adult cardiomyocytes can reproduce many of the responses observed in whole hearts, 2) caution should be used in adding lactate to an ischemic solution, and 3) it is important to stimulate contractile activity throughout ischemia to reproduce the effects of ischemia in whole hearts.

Contractile activity; lactate; calcium; single-cell system; ischemia-reperfusion injury

THE USE OF ISOLATED, SINGLE cardiomyocytes to study cell structure, contractile behavior, metabolism, and ionic flux has been of invaluable benefit to our understanding of cardiac function. The use of single cells to study these parameters allows for a more rigorous analysis of a problem without the confounding influence of the extracellular matrix, diffusion limitations inherent in the vascular compartment and the interstitial space, and, most importantly, the effects of other cell types. The latter is particularly significant in two ways: 1) noncardiomyocyte function may indirectly influence cardiomyocyte function, and 2) release of substances from noncardiomyocytes may affect cardiomyocyte function. For example, contraction of coronary arterial smooth muscle cells will influence cardiomyocyte contractile function through an indirect effect on blood flow and substrate supply to the heart cells. In addition, the release of nitric oxide from endothelial cells, for example, will also influence contractile behavior in cardiomyocytes when tissue preparations are used (28). The use of a homogeneous population of cells can remove these variables and allow the investigator to study the parameter of interest in isolation.

Ischemic heart disease is a major cause of mortality (17, 30). Reperfusion of an ischemic region also constitutes an important clinical problem of its own (13, 30). The vast majority of work on ischemia-reperfusion injury to the heart has been carried out using whole heart or cardiac tissue preparations (1, 6, 13, 17, 20, 22, 24, 26, 27, 29, 30, 35). The use of single cardiomyocytes in a model of ischemia-reperfusion injury would be advantageous. For example, diffusion problems have been suggested to influence drug inactivation of the Na\(^+\)/H\(^+\) exchanger during ischemia-reperfusion injury (22). Using single cells would remove this variable. Furthermore, few studies have accurately examined Ca\(^{2+}\) transients selectively in cardiomyocytes on a beat-to-beat basis during ischemia or reperfusion. Finally, and most importantly, single cells can be used with a variety of transfection strategies [gene transfer by microinjection (3), viral infection (25), or other techniques (19)] to induce overexpression of selected proteins. These cells may then be used to study the contribution of that protein to ischemia-reperfusion injury. Thus the development of an adequate model of ischemic injury in a single-cardiomyocyte system would represent an important advance in this field.

Single cells have been used to study hypoxic injury, metabolic injury, or simulated ischemic injury. However, in the vast majority of these studies, neonatal cardiomyocytes (2, 4, 25, 36–38) or quiescent adult cardiomyocytes (7, 8, 12, 15, 16) have been used. We hypothesized that there were important limitations inherent in these previous cell models of ischemic injury. First, neonatal hearts are well known to be significantly different from adult hearts with respect to cardiac metabolism, ion transport (26, 27), and their response to ischemia (26). The use of adult cardiomyocytes avoids this limitation, but the majority of these studies have employed quiescent cells (7, 8, 12, 15, 16). Because it is clear that ischemia has a very important metabolic component in the injury process (2, 7, 12, 13, 30), it is reasonable to hypothesize that the addition of contractile activity would significantly influence subse-
quent results. Three studies have used contracting adult cells (5, 14, 39), but it is unclear whether it is important to control cardiomyocyte contractile activity during ischemia-reperfusion. Furthermore, data collected from these studies were limited to the measurement of just one or two parameters in each study. The purpose of the present investigation, therefore, was to 1) fully characterize simulated, low-flow ischemia and reperfusion in a preparation of isolated, single, electrically stimulated cardiomyocytes from adult rat heart and 2) prove that it is important to control contractile activity in this model.

METHODS

Cardiomyocyte preparation. Single cardiomyocytes were isolated by enzymatic digestion of cardiac tissue from adult Sprague-Dawley rats (250–300 g), as described previously (18). These cells do not exhibit spontaneous contractile activity but will respond to electrical stimulation with appropriate contractile behavior for many hours, exclude trypan blue, demonstrate normal resting Ca^{2+} concentration and Ca^{2+} transients, and have resting membrane potentials of approximately −80 mV (18). Cells were isolated and then allowed to settle overnight on laminin-coated glass coverslips in medium 199 (GIBCO BRL, Burlington, ON, Canada) supplemented with 0.02% BSA, 50 U/ml penicillin, and 50 µg/ml streptomycin. The cardiomyocytes were used immediately on the following day for all studies. We have observed no changes in cell morphology or contractile behavior from the day of isolation to the following day.

Ischemia-reperfusion protocol. Cardiomyocytes mounted in a Leiden chamber were perfused at a rate of 1 ml/min with control perfusate heated to 37°C and bubbled extensively with 100% oxygen. The control perfusate contained 140 mM NaCl, 6 mM KCl, 1 mM MgCl_2, 1.25 mM CaCl_2, 6 mM HEPES (pH 7.4), 10 mM dextrose, and 0.02% BSA. After an initial period of equilibration, the perfusate was changed from the control perfusate to an ischemic solution, modified from Lukas and Ferrier (20). The ischemic solution and the control perfusate were similar, except for the following changes: 8 mM KCl, no dextrose, pH 6.0, and bubbled with 100% nitrogen gas for >45 min before the experiment was started. The Po_2 of the perfusate was reduced by −80%: 655 ± 12 and 137 ± 2 mmHg in the control (n = 6) and ischemic (n = 4) perfusates, respectively. In preliminary experiments, hearts were perfused with a control solution that was only depleted of oxygen by bubbling it with nitrogen, as described above. Tension generation was reduced to 15 ± 2% of control values (n = 3) after 15 min of perfusion. Clearly, therefore, this solution was hypoxic enough to induce significant changes in cardiac contractile performance in a Langendorff mode of perfusion. This is consistent with another report where a similar reduction in perfusate Po_2 resulted in a fivefold increase in lactate production and approximately fivefold decreases in myocardial oxygen consumption and mitochondrial energy production (31). In some cases, we included 0.1 mM sodium hydrosulfite in the perfusate to induce a depolarization of oxygen content of the perfusate with sodium hydrosulfite was 155 ± 4 mmHg (n = 5). The KCl concentration was increased slightly to mimic the in vivo condition (29, 34), the pH was reduced to approximate values for extracellular pH during ischemia in vivo (6, 31), and the decrease in substrate supply was implemented to mimic the hypoxic and metabolic inhibition that accompanies ischemia (2). The duration of ischemic perfusion was 90 min, followed by reperfusion with the control perfusate for 30 min.

Cardiomyocyte contractile performance. Active and passive cardiomyocyte contractile activity was measured as unloaded cell shortening by a video edge-detection system (Crescent Electronics, Sandy, UT) coupled to a Pulnix monochrome CCD camera. This camera captures data at a rate of 60 Hz. Cell shortening was monitored during electrical stimulation with platinum electrodes at a rate of 0.5 Hz with a duration of 100 ms. A microscope stage micrometer was used to calibrate the signal.

Cardiomyocyte viability. Cardiomyocyte integrity was measured as a function of the ischemic or reperfusion insult. Ethidium homodimer staining of cell nuclei was used as one method of assessing membrane integrity. This was carried out at the end of the experimental intervention using the Live/Dead assay kit (Molecular Probes, Eugene, OR). Cell morphology was used as another index of cellular integrity. Cardiomyocytes were visually assessed and counted on the full microscopic field with a ×10 lens. Cardiomyocytes were judged to be healthy if they had a normal rectangular rod shape, whereas damaged cells were subjectively determined by the presence of surface blebbing, rounded cell edges, or complete balling up of the cell.

Measurement of cellular pH and Ca^{2+} concentration. Intracellular Ca^{2+} transients were measured spectrofluorometrically using the Ca^{2+}-sensitive dye fura 2 (Molecular Probes), as described in detail elsewhere (9, 18, 23). In a typical experiment, cells already adherent to a coverslip were loaded with 2 µM fura 2-AM for 15 min at 37°C, washed, and then mounted in a Leiden chamber heated to 37°C with a Medical Systems PDMI-2 Open Perfusion Micro-Incubator (Green- vale, NY). This system was fixed on the stage of a Nikon Diaphot epifluorescence microscope, which was attached to a SPECT Fluorolog spectrophotometer, as described in detail elsewhere (23). Cardiomyocytes were alternately excited at 340- and 380-nm wavelengths with emission at 505 nm. Fluorescence was quantitated with photomultiplier tubes coupled to a Pentium computer. Calibration of the signal was carried out as described previously (9, 18, 23).

The protocol for the measurement of intracellular H^+ concentration was identical to that described above, except that 2',7''-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (Molecu- lar Probes) was employed as the fluorescent indicator dye. The dye was excited at 440 and 500 nm with emission at 525 nm. Detailed information is provided elsewhere (23). Fluorescent measurement of the generation of intracellular peroxides. The generation of intracellular oxidants was measured in cardiomyocytes with the fluorescent indicator carboxy-ylated dichlorodihydrofluorescein diacetate (carboxy- H_2DCFDA) (36) (Molecular Probes). Cells were loaded with 5 µM carboxy-H_2DCFDA-AM for 30 min at 37°C and then washed before placement in a measurement system as described above for Ca^{2+} and H^+. Cardiomyocytes were alternately excited at 340- and 360-nm wavelengths with emission at 505 nm. Images were captured in a Bio-Rad MRC 600 confocal microscope equipped with a Coherent argon ion laser. Control cells exhibit minimal fluorescence until the carboxy-H_2DCFDA is exposed to reactive oxygen species, which oxidizes the dye to yield the fluorescent product 2',7''-dichlorofluorescein.

Measurement of cardiomyocyte high-energy phosphate content. The cellular contents of ATP, ADP, and AMP were determined by HPLC by a modification of the method of Luo et al. (21). Briefly, cardiomyocytes were exposed to 0.4 M HClO_4 to extract the nucleotides and then centrifuged to remove the protein. This protein was solubilized in 5% SDS.
and 0.2 M NaOH and then quantitated with a modified Lowry protein assay, as described previously (18, 22). The extract supernatant was neutralized with 5 M KOH and 2.1 M KH₂PO₄, and this neutralized extract was dried after the KClO₄ precipitate was discarded. The sample was resuspended in water and injected into the HPLC system in a volume of 25 µl. Chromatography was carried out in a Waters HPLC system with a 150 × 4.6 mm Luna 5-µm C₁₈ analytic column fitted with a 30 × 4.6 mm guard column. The mobile phase was 99% 0.1 M sodium phosphate buffer (pH 4.0) with 1% acetonitrile flowing at a rate of 0.7 ml/min. Absorbance was monitored at 260 nm. Peaks were identified and quantitated by comparison to known quantities of ATP, ADP, and AMP standards dissolved in the same buffer as the neutralized extracts. Recovery was monitored with 8-bromo-AMP.

Measurement of cellular lactate. Cells were extracted after exposure for 20 min to the ischemia-mimetic solution, and intracellular lactate was determined as described in detail elsewhere (10).

Statistical analysis. A two-tailed Student's t-test or an ANOVA test was used to determine statistical difference. A Student-Newman-Keuls post hoc test was employed to identify statistical difference after the ANOVA test. Statistical significance was set at P < 0.05.

RESULTS

Cell survival was measured as a change in cell shape from the normal elongated, rod-shaped appearance to one of rounded edges, surface blebs, or balled-up configuration (Fig. 1). Cell survival was first investigated as a function of the duration of exposure to the ischemic solution (Fig. 2). Cells were stimulated throughout the experiment, and cell survival was monitored at the end of the 30-min reperfusion period. A 45-min exposure to the ischemia-mimetic solution resulted in a small loss of healthy cells in comparison to the preischemic period, but this was not significantly different from cells that were not exposed to the ischemic solution and were simply stimulated for 120 min. Longer durations of exposure to the ischemia-mimetic solution resulted in a significant loss of healthy cardiomyocytes. Only ~50% of the cells exhibited the normal rod-shaped appearance after 90 min of exposure to the ischemic solution. This was further reduced with ischemic durations of 120 and 150 min (Fig. 2).

Cell shortening (active and resting) was also investigated as a function of the duration of exposure to the ischemic solution. Cells were stimulated throughout the experiment, and cell shortening was followed throughout the reperfusion period. Data from many cells were collected during the postischemic reperfusion period, compiled, and presented in Fig. 3. Control cells did not exhibit a significant change in active shortening or in resting cell length during the last 30 min of a 120-min perfusion period (Fig. 3).
exposed to ischemia-mimetic solution for 45 min did not exhibit a change in resting cell length or active cell shortening during reperfusion. In comparison, the 90-min ischemia group exhibited a poorer recovery of active cell shortening and a significant decrease in resting cell length during reperfusion compared with the control group. The latter response is evidence that the cell had begun to passively contract in a manner similar to that observed with postischemic tissue preparations when resting tension or end-diastolic pressure increases (6, 13, 24, 30, 35). Similar results were observed in the cells exposed to an ischemic duration of 120 min (data not shown). This loss of cell contractile performance and decrease in resting cell length correlated well with the cell survival data shown in Fig. 2.

Cell survival was next investigated as a function of electrical stimulation throughout the ischemic period (Fig. 4). Cells were 1) not stimulated during ischemia or reperfusion, 2) stimulated only during reperfusion, or 3) stimulated throughout the 90-min period of ischemia. Cell survival was then measured at the end of the 30-min reperfusion period. Among cells stimulated to contract throughout ischemia and reperfusion, there were fewer healthy cells at the end of reperfusion than in preparations that were not stimulated (Fig. 4). The critical period for stimulation was the ischemic period, because if stimulation was instituted for just the reperfusion period, the effect on cell survival was similar to the group of cells that were not stimulated during reperfusion and ischemia. It was also important to control the frequency of stimulation. Reducing the frequency of stimulation from 0.5 to 0.2 Hz increased the percentage of healthy cells to control levels (Fig. 4).

Similar results were observed with respect to cell shortening as a function of stimulation during the ischemia-reperfusion periods (Fig. 5). Active cell shortening recovered fully if cells were stimulated only during reperfusion. It did not recover as well if cells were stimulated during both reperfusion and ischemia. Similarly, cells that were quiescent during ischemia and/or reperfusion exhibited no significant changes in resting cell length during reperfusion. However, cells stimulated during both conditions did exhibit a significant decrease in cell length.

Stimulation of cells during ischemia also affected cardiomyocyte metabolism. Perfusion of the cells with ischemic solution for 20 min resulted in a modest increase in cellular lactate in quiescent cells (Fig. 6). However, stimulation of the cells to contract during the ischemic period induced a further significant increase (~16-fold) in cellular lactate content over control conditions.

The composition of the ischemia-mimetic solution was an important variable for cell survival as well. Reducing the pH of the ischemic solution to 6.5 was not severe enough to induce any change in cell survival (Fig. 7). Similarly, if the ischemic solution contained glucose, the cells survived as well as control cells. If chemical hypoxia was induced with sodium hydrosulfite, the results were similar to those obtained by inducing hypoxia with a nitrogen-bubbled perfusate. Thus the ischemia-mimetic solution had to be depleted...
of energy substrates and have a relatively low pH (6.0) to induce changes in cell survival. The most striking effect on cell survival was produced by the addition of lactic acid to the ischemia-mimetic solution. Lactate at 10 mM reduced cell survival drastically. Similar results were observed with 20 mM lactate (data not shown).

We conducted a series of experiments on a limited number of variables to determine whether the changes in the composition of the ischemia-mimetic solution affected resting cell length and active cell shortening. As shown in Fig. 8, there was a good correlation between these parameters. Whenever cell survival was poor, the recovery of active cell shortening was significantly impaired as well. Conversely, whenever cell survival was good, cell shortening was also unaffected by the intervention.

Because our measure of cell viability was subjective, we also monitored cell viability with ethidium homodimer staining of cardiomyocyte nuclei. Cells with intact membranes stain green, whereas those with compromised membrane permeability characteristics allow access of the dye to the nuclei and emit a red fluorescence (Fig. 9). The two types of cells were quantified. The data confirm our subjective analysis of cell survival. Approximately 50% of the cells survived a 90-min ischemic period followed by 30 min of reperfusion (Table 1). Including 10 mM lactate in the ischemic solution resulted in an increase in the percentage of cells that exhibited red fluorescence, indicative of cell death (Fig. 9, Table 1). A longer exposure to the ischemia-mimetic solution (150 min) induced a greater percentage of cell death as well (Table 1). These values agree well with the subjective measurements presented in Fig. 1.

The mechanisms responsible for the changes in cell viability and contractile performance were investigated. The concentration of high-energy phosphates in the cardiomyocytes at the end of the postischemic period was measured. Absolute values for ATP content in cardiomyocytes in the present study were similar to those reported by others previously (2, 7, 12, 16) (Fig. 10). If our conditions did mimic an ischemic insult, we expected to observe a significant decline in ATP levels...
within the cardiomyocytes exposed to the ischemia-mimetic solution. As shown in Fig. 10, ATP levels were reduced by the ischemic insult. This decrease was augmented by the use of 10 mM lactate in the ischemic perfusate.

Others have demonstrated that oxygen-derived free radicals are generated during the ischemia-reperfusion insult in tissue preparations (17, 36). We used carboxy-H₂DCFDA as a fluorescent indicator of free radical generation during our experimental conditions. As shown in Fig. 11, fluorescent intensity within a cardiomyocyte loaded with carboxy-H₂DCFDA increased with exposure to an exogenously applied free radical-generating system (xanthine-xanthine oxidase). Before this exposure, fluorescent intensity of the dye was similar to background values. Exposure of the cells to superoxide dismutase did not affect the fluorescent signal within the cardiomyocyte. Because superoxide dismutase cannot gain access to the cell interior, this confirms the intracellular localization of the free radical signal. These data support the contention that this dye responds to oxygen-derived free radicals and are useful as an indicator of the presence of oxygen-derived free radicals. We used this dye, therefore, to determine whether our experimental conditions generate oxygen-derived free radicals, as has been observed by others in tissue settings. Figure 11 also shows representative recordings of carboxy-H₂DCFDA fluorescence from a cardiomyocyte exposed to the ischemia-mimetic solution. Note the large increase in cellular fluorescence that is indicative of the generation of free radicals during the ischemic period.

Intracellular pH (pHᵢ) is known to decrease significantly during ischemia in cardiac tissue preparations. This decrease in cellular pH can be as low as pH 5.7–6.3 (6, 32). We examined pHᵢ in cardiomyocytes exposed to the ischemia-mimetic solution for 90 min. A representative recording is presented in Fig. 12. There are several noteworthy points to consider. First, pHᵢ does not decrease for several minutes into the ischemic solution. This is not due to a limitation in the exchange rates for the solution perfusing over the cells. Instead, there appears to be an initial resistance to a drop in pHᵢ as the cardiomyocytes are exposed to the ischemia-mimetic solution. However, the cells do exhibit the characteristic drop in pH as the duration of ischemia proceeds beyond ~5 min. This decrease in pHᵢ is similar to that observed in vivo during ischemia. A second noteworthy feature is that the pHᵢ recovers relatively rapidly on reperfusion. We purposefully used a HEPES-based buffer in the perfusate to isolate the contribution of the Na⁺/H⁺ exchanger to changes in pHᵢ. The addition of a blocker of Na⁺/H⁺ exchange (10 µM dimethylamiloride) (30) to the perfusate prevented the postischemic recovery of pHᵢ (data not shown). Thus we may conclude that the recovery in pHᵢ during reperfusion is due to an activation of Na⁺/H⁺ exchange.

Changes in cellular Ca²⁺ during perfusion with the ischemia-mimetic solution and during reperfusion were also studied. A representative recording of fura 2 fluorescence from a single, beating cardiomyocyte is shown in Fig. 13. The results from a number of cells subjected to ischemia and reperfusion are presented in Fig. 14. Three features of the Ca²⁺ transient are noteworthy. First, the Ca²⁺ transients were dampened during the ischemic period (~30–60% of preischemic values) but remained surprisingly large in view of the minimal contractile activity observed. Cell shortening was 6 ± 4 and 0 ± 0% of preischemic activity at 10 and 20 min, respectively, after the ischemic perfusate was started. During these first 20 min of ischemic perfusion, pHᵢ remained above 6.7. Grynkiewicz and colleagues (9) previously showed that changes in pHᵢ to as low as 6.7 do not affect the fura 2 signal. Thus the majority of our Ca²⁺ transient recordings were not influenced by concomitant changes in pHᵢ. The cardiomyocyte apparently undergoes a futile cycling of Ca²⁺ through the cytoplasm during the ischemic period. Second, the Ca²⁺ transients recovered to control levels. Again, the magnitude of the recovery in the Ca²⁺ transient was far in excess of the recovery of contractile shortening exhibited in Fig. 3. Third, basal Ca²⁺ concentration increased steadily throughout the ischemic period. It remained elevated during the reperfusion phase and slowly decreased toward the end of reperfusion. This response in basal Ca²⁺ concentration is not dissimilar to that observed by others in perfused hearts during the ischemic and postischemic period (35).
Further studies were carried out on the effects of lactate on the cardiomyocytes to determine why it had such a deleterious effect (Fig. 7). There was a faster and larger decline in pH during the ischemic perfusion period in the presence of lactate than in its absence (Fig. 15). Even though the lactate was only included in the ischemia-mimetic solution, it continued to have effects on cellular pH during the reperfusion period. During the reperfusion phase the recovery in pH was very rapid and continued to rise beyond the preischemic pH level into the alkaline range. Basal Ca\(^{2+}\) concentration was also higher than normal during reperfusion, and the Ca\(^{2+}\) transient, if it recovered at all, was smaller in magnitude than if the lactate was not included during ischemia (data not shown). Most cells did not recover to allow a recording of the Ca\(^{2+}\) transient.

**DISCUSSION**

The present results demonstrate that single adult rat cardiomyocytes may be used in a simulated ischemic environment to generate data that mimic many of the responses of tissue preparations in situ or in vivo. This conclusion is based on eight independent lines of evidence. First, the cells were damaged in response to the ischemia-reperfusion protocol. This was demonstrated by the changes in cell morphology and sarcolemmal membrane permeability. Similar observations have been reported in ischemic hearts after reperfusion (30). Second, the severity of the damage was influenced by the duration of the ischemic insult. The longer the duration of ischemia, the greater the damage and dysfunction, as has been shown in tissue preparations. Third, the contractile response to our ischemia-mimetic solution was similar to that observed in hearts exposed to ischemia and reperfusion. The cells demonstrated a relatively rapid loss of contractile function after the onset of perfusion with the ischemic solution. The contractile activity recovered poorly after re instituted on the duration of the ischemic phase.

**Table 1.** Cell viability as assessed by ethidium homodimer staining in cardiomyocytes exposed to different experimental conditions

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Live Cells, % of Preischemia</th>
<th>Total Cell No. Counted</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.4 ± 1.2</td>
<td>433</td>
<td></td>
</tr>
<tr>
<td>Ischemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 min</td>
<td>47.4 ± 7.6</td>
<td>187</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>150 min</td>
<td>15.0 ± 3.2</td>
<td>116</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>90 min + 10 mM lactate</td>
<td>15.0 ± 1.4</td>
<td>66</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4–23 separate experiments involving cell numbers indicated above. Significance was evaluated vs. control sample.
This was accompanied by a rise in passive shortening, which is typical of a reperfused heart after a period of ischemia (6, 13, 24, 26, 27, 30, 35). Fourth, modifications in the substrate supply during ischemia limited recovery. Reducing the oxygen and glucose concentration of the ischemic perfusate resulted in poorer recovery of the cardiomyocytes. This is similar to tissue studies that have also shown a strong dependence on these substrates for cell survival during ischemia (8, 20). The reduction in perfusate PO2 used in the present study would be expected to decrease oxygen consumption of isolated perfused hearts by ~75%, depress left ventricular developed pressure by ~70%, and increase lactate production by nearly ninefold (31). The limited oxygen supply available to cells in the present study, therefore, represents a reasonable representation of the hypoxic component of the ischemic challenge. Fifth, pH, decreased to an appropriate level during perfusion with the ischemia-mimetic solution and recovered during the reperfusion phase. The values obtained are similar to those observed with NMR spectroscopy during ischemia-reperfusion of whole hearts (6, 32). Sixth, resting levels of Ca2+ increased during ischemia. This response is also consistent with tissue data (35). Seventh, compounds associated with the ischemia in whole hearts were also observed in our single-cell work. For example, we demonstrated the generation of reactive oxygen species within the cardiomyocyte during the perfusion with ischemia-mimetic solution. This is consistent with other studies that have documented the generation of oxygen-derived free radicals during the ischemic period (36). Eighth, ATP levels were depressed

Fig. 11. Generation of oxygen-derived free radicals in treated cardiomyocytes. Representative fluorescent recordings were derived from cardiomyocytes loaded with carboxylated dichlorodihydrofluorescein diacetate dye before experimental treatment (A and C) or in same respective cells after exposure to solution containing 2 mM xanthine-xanthine oxidase (0.03 U/ml) for 20 min (B) or after 40 min of exposure to ischemia-mimetic solution (D). Cellular fluorescent intensity decreased in cells exposed to ischemic perfusion for >50 min.

Fig. 12. Representative recording of intracellular pH in a cardiomyocyte exposed to ischemia-mimetic solution for 90 min and then reperfused with control perfusate for 30 min. Recording was interrupted at several time points to minimize photobleaching. 500/440 Ratio, ratio of fluorescence at 500 nm to that at 440 nm.

Fig. 13. Representative recording of intracellular Ca2+ concentration in a cardiomyocyte exposed to ischemia-mimetic solution for 90 min and then reperfused with control perfusate for 30 min. Recording was interrupted at several time points to minimize photobleaching. 340/380 Ratio, ratio of fluorescence at 340 nm to that at 380 nm.
in the cardiomyocytes by the ischemia-reperfusion protocol, as would be expected from tissue data (12, 30). The extent of the fall in ATP levels is not dissimilar to those observed in tissue during ischemia-reperfusion (35).

It is also important to acknowledge the limitations inherent in the use of the single cardiomyocyte for the study of ischemic injury. For example, the removal of other cell types allowed us to clearly define the function of the cardiomyocyte but does eliminate a potentially important modulating influence that may exist in vivo. Second, the ischemic solution is a mimetic solution and may not contain all aspects of the ischemic condition. The role of complement in ischemia-reperfusion injury and any vascular factors is not included. We have tried to approximate the composition of the ischemic perfusate as well as possible, but it is still an approximation. It is important to reiterate, however, that the decreased oxygen, glucose, and pH and elevated KCl have been demonstrated to occur in ischemic tissue (6, 20, 29, 32, 34) and are the most prominent changes in the composition of the extracellular fluid. Another limitation inherent in the present results concerns the cell shortening data. The active cell shortening measurements are carried out in an unloaded state. Thus we do not obtain measurements of maximal shortening. This is particularly relevant to the preischemic condition, where values clearly represent an underestimation of maximal shortening. In future experiments, this may be measured more accurately with a biaxial strain coupled to pacing. The cell shortening data in the present study, therefore, are merely indications of the contractile potential and may complement the data on resting cell length, Ca\(^{2+}\) transients, and cell viability to yield a more complete picture of the viability and functional integrity of these cells during postischemic reperfusion challenge.

Another potential limitation in our study concerns the constant flow used throughout the experimental period. This was done instead of employing a no-flow condition during the ischemic period, because we detected small quantities of oxidation products released from the stimulating electrodes. In a no-flow condition, these by-products accumulated during the lengthy times employed. This affected cardiomyocyte viability and reflected an artifact of the experimental protocol and not the ischemic insult per se. Thus we used a constant-flow condition in our protocol. We are confident that this removed the influence of the small amounts of oxidation products released from the stimulating electrodes, because electrically stimulated cardiomyocytes functioned without problems for 4–5 h under normal perfusion conditions. However, the use of a constant-flow protocol did result in the removal of some metabolic end products from our ischemic cells. Our model, therefore, is best described as a model of low-flow ischemia. It is important to recognize that most ischemic lesions in vivo are not no-flow conditions but involve a reduction in flow.

These data represent the most detailed characterization to date of the effects of an ischemic solution on cardiomyocyte contractile function, viability, energy status, and ionic homeostasis. The work has generated data supporting the model and its similarity to the tissue setting and has also yielded new data of interest. For example, we have demonstrated that contractile activity in single cardiomyocytes is important during ischemia-reperfusion injury. Cells that were electrically stimulated exhibited more frequent and severe dysfunction than those that were not stimulated. The rate of stimulation and its duration (ischemia-reperfusion vs. reperfusion) were important variables to control. A lack of contractile activity in single cardiomyocytes is known to reduce oxygen cost by \(\sim 60\%\) (33). These data emphasize the importance of stimulating adult cells during a simulated ischemic condition and the significance of the cellular metabolic status and the metabolic stress placed on the cardiomyocyte during this pathology.

If the cell is not actively contracting during ischemia in response to the electrical stimulation, then how is...

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**Fig. 14.** Intracellular Ca\(^{2+}\) in cardiomyocytes exposed to ischemia-mimetic solution for 90 min and then reperfused. Ischemia was initiated at time 0, and reperfusion was started at 90 min. Values are means ± SE for 12 cells.

**Fig. 15.** Intracellular pH in cardiomyocytes exposed for 90 min to an ischemia-mimetic solution with and without 10 mM lactate and then reperfused with control perfusate for 30 min. Values are means ± SE for 5 cells. *P < 0.05 vs. ischemia.
the energy being consumed? Part of the metabolic cost of contractile activity involves the regulation of intracellular Ca\(^{2+}\) concentration (7). Although the intracellular Ca\(^{2+}\) transients were rapidly inhibited by the ischemia-mimetic solution, we did find a surprisingly high Ca\(^{2+}\) transient throughout the ischemic period, when one considers that contractile activity was negligible. This suggests that although the cell may be inactive in a contractile sense, the cardiomyocyte still responds to electrical stimulation with a relatively large amount of Ca\(^{2+}\) cycling through the cytoplasm. Because the Ca\(^{2+}\) transient consumes a significant amount of energy (7), this would have implications for the energy status of the cell.

Another interesting finding in our study was the strong sensitivity of the cardiomyocytes to lactate in the ischemia-mimetic solution. Many previous investigations have included 10–20 mM lactate in their ischemia-mimetic solutions (5, 13, 20, 37). On the basis of the present results, this would not appear to be an optimal compound to include in the ischemia-mimetic solution. In the present study, cells exposed to the ischemia-mimetic solution with lactate included responded with a faster and more severe decrease in pH, and, ultimately, had a greater chance of undergoing cellular necrosis than cells exposed to an identical ischemia-mimetic solution in the absence of lactate. Others have observed similar effects of short-term exposure to lactate during control perfusion conditions (32). However, the present data are in conflict with the conclusions of Geisbuhler and Rovetto (8), who found that lactate did not influence anoxia-reoxygenation damage in adult cardiomyocytes. The conflict in results could be due to the difference in intervention used (simulated ischemia vs. anoxia) or the contractile status of the cells (quiescent in the previous work vs. electrical stimulation in the present study). However, we have tested the effects in quiescent cells exposed to the ischemic solution containing 10 mM lactate and found deleterious effects similar to those observed in the stimulated cells (data not shown). This would suggest that the former possibility is the most likely one. The effects of lactate in the present study were likely due to an entry of H\(^{+}\) into the cardiomyocyte through the lactate-H\(^{+}\) cotransport pathway (32). Consistent with previous work, the pH data in the present study suggest that the lactate-H\(^{+}\) cotransporter in cardiomyocytes is capable of rapid transport of lactate and H\(^{+}\) into the cell. It would appear, therefore, that the inclusion of lactate in the ischemia-mimetic solution must be employed with considerable caution if one is to use this system as a model for ischemia in hearts in vivo. The addition of 10 mM lactate to the ischemic solution induces a severe, irreversible stress on the cells. It may be more appropriate to allow the cell to generate intracellular lactate on its own rather than induce an abnormally large acidification, if one is to mimic the tissue conditions.

The present results demonstrate that this model is a reasonable cellular representation of ischemia-reperfusion in the heart. Our work represents the first evaluation of a comprehensive set of parameters in a single, beating adult cardiomyocyte model of simulated ischemia and reperfusion. A large number of the effects of ischemia in cardiac tissue were replicated in this single-cardiomyocyte system. It represents a significant improvement over neonatal and quiescent adult cardiomyocyte models of ischemic challenge. It will be an excellent model for testing hypotheses that are suited to single-cell settings. The most intriguing of these includes the use of transfected cells, where proteins of interest to the ischemic field may be examined for their potential contribution to ischemia-reperfusion injury. One of the biggest problems facing gene therapy today in humans or animals is the immune response generated by the introduction of the transfection vehicle. The ischemic model tested in the present study avoids this complication and should allow us to obtain valuable preliminary information about the role of a specific protein in ischemia-reperfusion injury while the approach of gene therapy in the whole body awaits perfection.

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REFERENCES

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