Cellular Energetics and the Oxygen Dependence of Respiration in Cardiac Myocytes Isolated from Adult Rat*

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The oxygen dependence of mitochondrial respiration was investigated using suspensions of mitochondria and quiescent ventricular myocytes isolated from adult rat hearts. A new optical method was used to determine oxygen concentration in the suspending media. The $P_{O_2}$/ADP ratio for respiration and coupled mitochondria averaged 1.04 ± 0.02, but was increased to 2.57 ± 0.02 by the addition of succinate to the substrate mixture. This value was decreased to less than 0.01 when the ATP/ADP ratio was decreased with uncoupler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone. The $P_{O_2}$ value in resting myocytes was 2.5 ± 0.13 $\mu$M at a V_max of 13.3 ± 1.38 nmol O_2/g dry weight/min. During resting conditions, the creatine phosphate/creatine and ATP/ADP ratios were high in these cells, 6.8 ± 1.1 and 1131 ± 185, respectively. Addition of 1 mM Ca^{2+} to the suspending media increased the $P_{O_2}$ by 50% whereas respiration rose by only 10%. Respiratory rate was increased up to about 10-fold by uncoupling the cells, but the $P_{O_2}$ increased by less than 3-fold. When these uncoupled cells were inhibited with Antymol to lower the rate of oxygen consumption to that of resting cells, the $P_{O_2}$ fell to 1.26 ± 0.14 $\mu$M. Diffusion models indicate that in resting myocytes, the oxygen concentration difference from sarcolemma to core cell was approximately 1.84 $\mu$M with an additional difference of about 0.27 $\mu$M attributed to the unstirred layer of media surrounding each cell. The intracellular oxygen diffusion coefficient in myocytes was calculated to be 3.0 $\times$ 10^{-5} cm^2/s. The results show that the oxygen dependence of respiration is modulated by the cellular metabolic state. At near maximal levels of respiration or on recovery from hypoxic episodes, oxygen diffusion may become an important determinant of the oxygen dependence of myocardial respiration.

In cardiac myocytes, ATP turnover is high, relative to other cell types, due largely to the continuous hydrolysis of this high energy phosphate to sustain contractile tension during the cardiac cycle. Since mitochondrial oxidative phosphorylation is the primary mode of ATP synthesis in the heart, oxygen must be supplied to its cells, and in particular to the mitochondria, at a rate consistent with the rate of ATP utilization. Within the cell, oxygen delivery to the cytochrome oxidase occurs in response to the diffusive fluxes of oxymyoglobin and dissolved oxygen. These fluxes are in turn responsive to their respective differences in concentration between the sarcolemmal membrane and the mitochondria. The nature of intracellular oxygen concentration differences has been the subject of considerable controversy (1) and is important because of their potential effects on cellular energetics and cellular function. This is especially true for cardiac myocytes which have stringent ATP requirements.

In tissue, a "critical" oxygen concentration has been assumed to be established by the K_c of the cytochrome oxidase and the oxygen pressure difference between the capillaries and the mitochondria. If, as suggested by some workers (2-4), cytochrome oxidase is completely saturated until the oxygen pressure is less than 1 $\mu$M, the critical value would only be reached during periods of marked reductions of oxygen delivery to cells. Within cardiac and skeletal myocytes, there exists an auxiliary process for enhancing and facilitating uniform oxygenation, i.e. myoglobin. It has been reported that the concentration of myoglobin in muscle cells is, in part, determined by the distance through the cell that oxygen must diffuse to reach the mitochondria (5). This is borne out by the finding that red skeletal myocytes contain approximately twice the concentration of myoglobin and have a 2-fold greater diffusion radius for oxygen than do cardiac myocytes (6). That a mechanism, such as the facilitated diffusion of oxygen by myoglobin, has evolved to ensure a constant supply of oxygen to the respiratory chain suggests that the process of mitochondrial oxidative phosphorylation is highly sensitive to physiological levels of oxygen.

We have demonstrated previously that, in isolated mitochondria (7) and in cells without myoglobin (8), mitochondrial oxidative phosphorylation is dependent on oxygen at concentrations found in the physiological range. These findings would argue against a "critical" value and indicate that there exists a wide range of oxygen values that influence the metabolic state of the tissue. The present investigation was carried out to characterize the oxygen dependence of respiration in isolated cardiac myocytes, determining both the oxygen dependence of the mitochondria in situ and the diffusion-induced difference in oxygen concentration between the extra cellular medium and the mitochondria.

EXPERIMENTAL PROCEDURES

Materials—Collagenase (type II) was obtained from Worthington. Bovine serum albumin (BSA), Fraction V was purchased from ICN.
Immunobiologicals (Lisle, IL). Enzymes and reagents were acquired from either Sigma or Boehringer Mannheim unless stated otherwise. Isolation of Cardiac Myocytes—Calcium-tolerant ventricular myocytes were isolated from hearts of adult rats (250–300 g) according to the procedure of Wittenberg and Robinson (9). In brief, hearts from Sprague-Dawley rats were perfused retrogradely at 72 cm of H$_2$O and 37°C with a low calcium Hepses-Ringer buffer supplemented with 14 mM glucose and insulin (10 milliunits/ml). The perfusate was gassed with 100% O$_2$. After 3 min, the hearts were perfused via recirculation with the same medium plus 0.1% collagenase (231 units/mg) at a constant flow of 6 ml/min/heart. The ventricles were minced and incubated for 10 min in similar medium containing 0.15% collagenase, 0.7% BSA, and 0.3 mM CaCl$_2$ using a Dubnoff metabolic shaker water bath at 37°C. Three to four additional incubations were necessary to harvest the entire cell population. The cells were washed, separated by density gradient centrifugation in isotonic Percoll, and resuspended in Hepses-Ringer buffer containing 0.7% BSA and 0.3 mM CaCl$_2$.

Cells were used immediately following morphological analysis of viability and were maintained at room temperature during the experiments. The number of quiescent rod-shaped cells ranged from 70 to 90% within a total population of 5–9 × 10$^6$ cells. A hemocytometer was used to evaluate the morphometry of the cells.

Isolation of Mitochondria—Mitochondria were prepared from hamster heart myocytes using the isolation procedure of Fuller et al. (10). Immediately following decapitation of the animal, the heart was excised and trimmed free of the atria and great vessels. The ventricles were minced in ice-cold isolation medium (0.225 mM mannitol, 75 mM sucrose, 1.0 mM EGTA, and 10 mM MOPS, pH 7.4), briefly exposed to the proteolytic enzyme preparation, Nagase (Enzyme Development Corp., New York), and homogenized with a Polytron. The mitochondria were separated from the remainder of the broken cells using density gradient centrifugation. Respiratory control ratios were from 9 to 14 in the presence of 5 mM glutamate and 5 mM malate.

Measurements of Oxygen Consumption—Mitochondria were suspended in Hepes-Ringer buffer containing 0.7% BSA and 0.3 mM CaCl$_2$. Cells were used immediately following morphological analysis of viability and were maintained at room temperature during the experiments. The number of quiescent rod-shaped cells ranged from 70 to 90% within a total population of 5–9 × 10$^6$ cells. A hemocytometer was used to evaluate the morphometry of the cells.

Isolation of Mitochondria—Mitochondria were prepared from hamster heart myocytes using the isolation procedure of Fuller et al. (10). Immediately following decapitation of the animal, the heart was excised and trimmed free of the atria and great vessels. The ventricles were minced in ice-cold isolation medium (0.225 mM mannitol, 75 mM sucrose, 1.0 mM EGTA, and 10 mM MOPS, pH 7.4), briefly exposed to the proteolytic enzyme preparation, Nagase (Enzyme Development Corp., New York), and homogenized with a Polytron. The mitochondria were separated from the remainder of the broken cells using density gradient centrifugation. Respiratory control ratios were from 9 to 14 in the presence of 5 mM glutamate and 5 mM malate.

Measurements of Oxygen Using Phosphorescence—The method by which phosphorescence was used to measure oxygen consumption has been described previously in detail (7, 11). In brief, oxygen concentration in the suspending medium was measured by its quenching of the phosphorescence arising from palladium coproporphyrin (Porphyrin Products, Logan, UT). A xenon flash lamp was used to excite the luminophore to its triplet state with the light of wavelengths between 380 and 440 nm. The lifetime of the emitted light (>635 nm) was used to calculate the oxygen concentration from the Stern-Volmer relationship (for more detail, refer to Ref. 5). The quenching constant was found to be 1.4 × 10$^{-3}$ M$^{-1}$ s$^{-1}$ for the experimental conditions and was unaffected by addition of reagents to the cuvette. The assay cuvette was of glass construction (total volume = 2.4 ml) and contained a small Teflon-coated stirring bar to maintain the mitochondria or cells in suspension. The cuvette was mounted on a magnetic stirrer. Aliquots of suspensions of either mitochondria or cardiocytes were added to the cuvette containing the respective assay medium and diluted to the concentrations noted in the legends of the figures or tables. A ground glass stopper was used to eliminate the gas phase. This stopper also provided access to the assay medium via a central hole (1.3 mm, internal diameter) for additions during the experiment.

For measurements of oxygen consumption using isolated mitochondria, the assay medium consisted of 0.13 M KCl, 0.2 mM EGTA, 0.015 M MOPS, and 0.3% bovine serum albumin (w/v), pH 7.5. Oxygen determinations using isolated cells, the assay medium was that used to suspend the cells. The oxygen probe, palladium coproporphyrin (2 μM), and catalase (260 unit/ml, Sigma) were added to the respective assay buffers. Addition of a small aliquot of H$_2$O$_2$ (0.2 mM stock) which was rapidly converted to O$_2$ and water provides a simple method for introducing oxygen into the assay system.

The sample was stirred at a rate sufficient to keep the cells suspended and yet not so rapidly as to compromise the viability of the cells. The rate necessary for keeping the cells fully suspended was measured by the redox state of the intramitochondrial pyridine nucleotides (i.e. the NAD$^+$/NADH ratio) using the equation,

$$K_{eq} = \frac{[NADH][\alpha-KG][NH_3]}{[NAD^+][Glut]} = 3.87 \times 10^{-3}$$

FIG. 1. Oxygen consumption by mitochondria isolated from rat heart. A, mitochondria were suspended at a cytochrome c concentration of 0.2 μM in media described under "Experimental Procedures." Oxygen consumption was initiated by the addition of 5 mM glutamate, 5 mM malate, 5 mM succinate in the presence of 0.8 mM ATP. In this representative sample, oxygen consumption was linear above 8 μM oxygen so that only the data in the oxygen-dependent region are plotted (closed circles). B, oxygen consumption by uncoupled mitochondria. Mitochondria were suspended at a concentration of 0.11 mg/ml of media. Sufficient uncoupler, FCCP, was added to maximally stimulate respiratory rate. The data (closed circles) from a representative example are plotted from 0 to 5 μM oxygen for comparison to coupled values shown in A. The solid lines in A and B represent the best fit of the data to the equation shown under "Experimental Procedures."
The dependence of maximal respiratory rate and the $P_{50}$ for oxygen on the concentration of mitochondria and metabolic state

Mitochondria were isolated from rat heart as described under "Experimental Procedures" and suspended in medium containing 130 mM KCl, 15 mM MOPS, 0.2 mM EGTA, 0.8 mM ATP with either 5 mM glutamate, 5 mM malate or 5 mM glutamate, 5 mM succinate, 5 mM malate as the respiratory substrate. The concentration of cytochrome $c$ in the cuvette was $0.2 \pm 0.03 \mu M$ (1 x) and was increased by 2- and 4-fold. All values represent means ± S.E. for the number of experiments noted in parentheses.

<table>
<thead>
<tr>
<th>Mitochondrial concentration</th>
<th>Glutamate/malate</th>
<th>Glutamate/succinate/malate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{max} \mu M/s$</td>
<td>$P_{so} \mu M$</td>
</tr>
<tr>
<td>1 ×</td>
<td>0.093 ± 0.007 (3)</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>2 ×</td>
<td>0.214 ± 0.012 (6)</td>
<td>0.44 ± 0.04</td>
</tr>
<tr>
<td>4 ×</td>
<td>0.465 ± 0.077 (3)</td>
<td>0.53 ± 0.08</td>
</tr>
</tbody>
</table>

where $[a-KG]$, $[NH_3]$, and $[Glut]$ are the measured intracellular concentrations of α-ketoglutarate, ammonia, and glutamate, respectively.

Data Presentation—All tabular data are presented as the means ± S.E. for the number of independent experiments given in the parentheses ($n$). An independent experiment is defined as the average of several measurements (2-4) obtained from a single preparation of cells. Data provided in the graphical presentations can be considered typical for a given set of experiments.

RESULTS

The Effect of Oxygen Concentration on the Respiration of Suspensions of Isolated Mitochondria—The rate of respiration by suspensions of mitochondria isolated from rat heart was unaffected by the concentration of molecular oxygen when its concentration was greater than about 8-10 μM. Fig. 1A shows that as oxygen was depleted via oxidative phosphorylation, the rate of respiration became markedly compromised below about 24 μM oxygen is displayed. The closed circles represent data obtained from a typical experiment, and the best fit of the data to the equation shown under "Experimental Procedures" is shown by the solid line.

![Fig. 2. Oxygen dependence of respiratory rate in quiescent myocytes isolated from rat heart. Myocytes were suspended at a concentration of 1.3 mg dry weight/ml of Krebs-Hepes buffer containing 0.7% bovine serum albumin, 0.3% CaCl$_2$, 2 μM palladium coproporphyrin and 260 units/ml catalase. At [O$_2$] above 25 μM, oxygen consumption fell progressively until the available oxygen was completely utilized, resulting in a P$_{50}$ of about 0.1 μM. When raising the concentration of mitochondria in the assay cuvette by 2- and 4-fold, the consumption of oxygen fell progressively until the available oxygen was completely utilized, resulting in a P$_{50}$ of about 0.1 μM. When raising the concentration of mitochondria in the assay cuvette by 2- and 4-fold, the consumption of oxygen rose in parallel (Table I). This change in oxygen utilization occurred for two separate combinations of substrate, glutamate/malate, or glutamate/malate plus succinate. In both cases, the value of P$_{50}$ was not markedly affected by the rise in oxygen utilization. In general, the respiratory rate was independent of oxygen concentration above about 20-25 μM. Below this value, oxygen consumption progressively diminished as oxygen concentration in the medium decreased. For this example, only the data below 24 μM are shown in which maximal velocity of respiration was 0.17 μM/s with a P$_{50}$ for oxygen of 2.41 μM. Data obtained from several preparations of ventricular cells are displayed in Table II. The mean P$_{50}$ for cells suspended in a low calcium medium was 2.23 μM ± 0.13 at a V$_{max}$ of 0.28 μM/s. When the cells were reoxygenated and the concentration of calcium in the medium was then raised to physiological levels (1.3 mM) the P$_{50}$ increased by about 96%. This increase was brought about with minimal elevation, about 10%, in V$_{max}$.

The Effect of Oxygen on Respiration by Suspensions of Cardiac Myocytes—A typical example of the oxygen dependence of respiration by suspensions of quiescent ventricular cells isolated from rat heart is shown in Fig. 2. In general, the respiratory rate was independent of oxygen concentration above about 20-25 μM. Below this value, oxygen consumption progressively diminished as oxygen concentration in the medium decreased. For this example, only the data below 24 μM are shown in which maximal velocity of respiration was 0.17 μM/s with a P$_{50}$ for oxygen of 2.41 μM. Data obtained from several preparations of ventricular cells are displayed in Table II. The mean P$_{50}$ for cells suspended in a low calcium medium was 2.23 μM ± 0.13 at a V$_{max}$ of 0.28 μM/s. When the cells were reoxygenated and the concentration of calcium in the medium was then raised to physiological levels (1.3 mM) the P$_{50}$ increased by about 96%. This increase was brought about with minimal elevation, about 10%, in V$_{max}$.

The effect of the metabolic energy state on the P$_{50}$ value of mitochondrial respiration was demonstrated by uncoupling phosphorylation of ADP from the oxidation of substrate. In this case, when maximal velocity was 0.033 and 0.08 μM/s, the mean P$_{50}$ was 0.06 ± 0.01 μM ($n = 4$). This value was near the limits of the method and represented only an upper limit. By comparison to the coupled condition, it can be seen in Fig. 1B that uncoupling the mitochondria shifted the oxygen dependence to the lower oxygen concentrations and decreased the value of P$_{50}$. This change was most apparent when the rate of respiration was equivalent to the coupled rate. Because the rate of respiration was markedly increased by uncoupling the mitochondria, the mitochondrial suspension was diluted immediately prior to its addition to the assay cuvette. The process makes it possible to obtain the number of data points necessary to mathematically resolve P$_{50}$ values in the region of low oxygen. Even with these precautions, the value of P$_{50}$ obtained in the uncoupled state is obscured by the limits of the measuring system, i.e. it is necessary to have at least 5 points in the oxygen-dependent region of the curve. At a respiratory rate of 0.15 μM/s and 2.5 points/s (our current limit), the lowest value of P$_{50}$ which could be resolved was about 0.1 μM.
The effect of the energy state on the $P_{50}$ for oxygen in isolated cardiocytes

All values represent means ± S.E. for (n) number of experiments. The aliquot of cell suspension in the cuvette was equivalent to 3.5 ± 0.4 mg, dry weight, of cells. After reoxygenation, the calcium concentration in the assay medium was raised from 0.3 to 1.3 mM. Maximal rates of respiration and a lowering of the energy state were then obtained with addition of the uncoupler, FCCP. Addition of Amytal was used to decrease the uncoupled rate of respiration to control values.

<table>
<thead>
<tr>
<th>Cell concentration</th>
<th>Control</th>
<th>1.3 mM Ca$^{2+}$</th>
<th>+FCCP</th>
<th>+Amytal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ ($\mu$M/s)</td>
<td>0.277 ± 0.02 (7)</td>
<td>0.317 ± 0.03 (6)</td>
<td>2.92 ± 0.22 (6)</td>
<td>0.244 ± 0.06 (5)</td>
</tr>
<tr>
<td>$P_{50}$ ($\mu$M)</td>
<td>2.23 ± 0.13</td>
<td>3.47 ± 0.21</td>
<td>9.53 ± 0.68</td>
<td>1.25 ± 0.14</td>
</tr>
</tbody>
</table>

Fig. 3. Oxygen dependence of respiration in uncoupled myocytes. The conditions of incubation were as described in the legend of Fig. 3, except that sufficient uncoupler, FCCP, was added to maximally stimulate respiration plus an additional 50%.

The oxygen concentration difference between the extracellular medium and the mitochondria. This is clearly defined in Fig. 4, where the uncoupled rate of respiration has been slowed by further treating the cells with Amytal, a respiratory chain inhibitor. In the latter condition, respiratory rate was equivalent to that observed on the initial cycle, and the $P_{50}$ was 1.3 μM. This value was markedly less than that observed for the resting cells at the same respiratory rate.

In the experiments using isolated mitochondria, it was shown that the concentration of mitochondria in the assay cuvette had no significant effect on the value of $P_{50}$ in the coupled state. Since the number of cells in the assay cuvette may alter the concentration of metabolites in the suspending medium, it was important to determine whether the level of cellular respiration modified the $P_{50}$ value. Table IV shows that raising the number of cells over a 3-fold range resulted in a concomitant change in oxygen utilization from 0.08 ± 0.01 to 0.32 ± 0.05 μM/s. The value of $P_{50}$, however, remained about 2 μM. The respiratory rate and $P_{50}$ were slightly less at the lowest concentration of cells (1 ×). This suggests that the cells may not have been as stable in very dilute suspensions as at higher concentrations. Moreover, by increasing the concentration of extracellular calcium from 0.3 to 1.3 mM, the $P_{50}$ rose at each cell concentration.

**Table II**

The effect of the energy state on the $P_{50}$ for oxygen and on the respiratory rate in isolated cardiocytes

All values represent means ± S.E. for (n) number of experiments. The aliquot of cell suspension in the cuvette was equivalent to 3.5 ± 0.4 mg, dry weight, of cells. After reoxygenation, the calcium concentration in the assay medium was raised from 0.3 to 1.3 mM. Maximal rates of respiration and a lowering of the energy state were then obtained with addition of the uncoupler, FCCP. Addition of Amytal was used to decrease the uncoupled rate of respiration to control values.

<table>
<thead>
<tr>
<th>Cell concentration</th>
<th>Control</th>
<th>1.3 mM Ca$^{2+}$</th>
<th>+FCCP</th>
<th>+Amytal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ ($\mu$M/s)</td>
<td>0.169 ± 0.02</td>
<td>0.163 ± 0.01</td>
<td>0.053 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>$P_{50}$ ($\mu$M)</td>
<td>2.1 ± 0.16</td>
<td>1.15 ± 0.24</td>
<td>0.059 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

**Table IV**

The effect of respiration on the $P_{50}$ for oxygen in isolated cardiocytes

Values represent means ± S.E. for three experiments. Control values were obtained with cells incubated in medium containing 0.3 mM calcium. Addition of calcium (final concentration = 1.3 mM) occurred after reoxygenation of this medium with H$_2$O$_2$.

<table>
<thead>
<tr>
<th>Cell concentration</th>
<th>Control</th>
<th>Plus calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ ($\mu$M/s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P_{50}$ ($\mu$M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 ×</td>
<td>0.080 ± 0.001</td>
<td>1.67 ± 0.07</td>
</tr>
<tr>
<td>2 ×</td>
<td>0.238 ± 0.003</td>
<td>2.61 ± 0.17</td>
</tr>
<tr>
<td>3 ×</td>
<td>0.324 ± 0.05</td>
<td>2.35 ± 0.27</td>
</tr>
</tbody>
</table>

**Fig. 3**. Oxygen dependence of respiration in uncoupled myocytes. The conditions of incubation were as described in the legend of Fig. 3, except that sufficient uncoupler, FCCP, was added to maximally stimulate respiration plus an additional 50%.

**Fig. 4**. Oxygen dependence of respiration in uncoupled inhibited myocytes. The same cells shown in Fig. 3 were reoxygenated and treated with Amytal at a concentration sufficient to decrease the uncoupled rate of respiration to control values.
value of $P_{so}$ for mitochondrial respiration was, in large part, determined by the metabolic conditions imposed by the local milieu. We therefore evaluated the metabolic state of the cells associated with the above experimental protocols. In Table V, it can be seen that the [ATP]/[ADP] and [creatine phosphate]/[creatine] ratios were large in these quiescent cells and corresponded to a [ATP]$_{mit}$/[ADP]$_{mit}$ of 1051 ± 185, calculated using the equilibrium constant for the creatine phosphokinase reaction (17). No change in the these ratios occurred when the extracellular calcium was raised from 0.3 to 1.3 mM, but there was a trend for a decrease in the intramitochondrial [NAD$^+$]/[NADH] ratio evaluated by assuming near equilibrium for glutamate dehydrogenase; from 15.4 ± 5.6 to 14.1 ± 6.6 ($n = 3$) was found. Treatment of the cells with uncoupler for 1 min markedly decreased the ATP/ADP and creatine phosphate/creatine ratios and increased the [lactate]/[pyruvate] ratio in a dose dependent manner. The latter data demonstrate the efficacy of the uncoupler treatment on the energy state of the cells used in the oxygen dependence experiments.

DISCUSSION

Among the many types of cells in the body, the oxidative capacity of cardiac myocytes, at least in situ, is perhaps the greatest, consistent with the energy demand imposed by the need for continuous cycling of the contractile proteins within these cells. Because of this large requirement for oxygen, isolated cardiac myocytes are an important model for study of the factors affecting the oxygen dependence of respiration. Two main findings resulted from the present investigation. 1) The oxygen dependence of respiration by mitochondria isolated from cardiac muscle is a function of the metabolic conditions, i.e. energy state and substrate availability, established by the medium in which they are suspended. 2) The oxygen dependence of mitochondrial respiration in situ is dependent upon both the cellular metabolic state and the oxygen concentration difference between the extracellular medium and the mitochondria. This difference is a function of the respiratory rate. The experimental data have been used to calculate the respiration-induced oxygen concentration difference according to equations describing oxygen diffusion and reaction in cylindrical cells (see Appendix). The observations are consistent with the respiratory rate being primarily a function of metabolic parameters for resting cells (see also Refs. 7, 8, and 18) with relatively little dependence on

intracellular oxygen diffusion. As the metabolic activity (respiratory rate) is elevated, there is a progressive increase in the contribution of oxygen diffusion until, at maximal respiratory rates, diffusion limitations are of major importance in determining the oxygen dependence of respiration.

In the present investigation, the rate of respiration by suspensions of mitochondria isolated from cardiac muscle began to decrease as the oxygen concentration declined below about 8 μM (a $P_{so}$ of 0.5–0.7 μM), whereas in cells the region of oxygen dependence began at higher oxygen concentrations, about 20 μM (a $P_{so}$ of 2.3 μM). The difference in the profiles of oxygen utilization was due to two factors: 1) the metabolic state of the mitochondria was substantially different, with the mitochondrial pyridine nucleotides having been more reduced and the energy state having been lower in the suspensions of isolated mitochondria, and 2) in cells, oxygen had to diffuse through a substantial distance from the suspending medium to the mitochondria where oxygen was reduced to water. Thus, for cells but not for mitochondria, there are significant diffusion-induced oxygen concentration differences between the extracellular medium, where it is measured, and the mitochondria. Both the metabolic oxygen dependence and the diffusion-induced oxygen concentration must be considered in order to fully understand the interplay between myocardial oxygenation and cellular energetics.

At concentrations of oxygen greater than 240 μM, the phosphorylation state ratio ([ATP]/[ADP][Pi]) of the quiescent myocytes was high as compared with that found in other types of cells in suspension (for review refer to Ref. 19). The authors are not aware of any reports within the literature that provide values of the phosphorylation potential for cardiac myocytes isolated from mammalian species (creatine concentrations are not normally measured, precluding such a calculation). That the phosphorylation potential is greater than in other cells may be due to the large oxidative capacity of the heart. For comparison, however, in isolated nonjecting rat heart perfused at 37 °C and electrically paced at 300 beats/min, the creatine phosphate/creatine ratio is about 4-fold less than that observed for the isolated myocytes, 1.2–1.4 versus 6.2 (20, 21). Given the quiescent nature of the isolated cells, it might have been expected that the phosphorylation potential would be higher than that for the perfused heart. Oxygen consumption was, however, comparable for perfused heart and isolated cells (16 versus 13 nmol of O$_2$/g, dry weight-min$^{-1}$, respectively) suggesting that the differences in energetics may in part be attributed to the higher concentrations of oxygen in the latter preparation. The median intracellular oxygen concentration of individual myocytes has been measured in rat subepicardium using myoglobin cryospectroscopy and found to be about 10 μM, varying by less than 3 μM within

<table>
<thead>
<tr>
<th>ATP</th>
<th>ADP</th>
<th>ATP/ADP</th>
<th>CrP</th>
<th>Cr</th>
<th>CrP/Cr</th>
<th>Lac</th>
<th>Pyr</th>
<th>Lac/Pyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.2 ± 1.4</td>
<td>2.0 ± 0.2</td>
<td>11.6 ± 0.8</td>
<td>25.2 ± 0.8</td>
<td>4.1 ± 0.6</td>
<td>6.5 ± 1.1</td>
<td>93.8 ± 6.0</td>
<td>19.8 ± 3.9</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>20.1 ± 1.4</td>
<td>2.1 ± 0.4</td>
<td>10.4 ± 1.6</td>
<td>24.5 ± 1.0</td>
<td>4.2 ± 0.8</td>
<td>6.76 ± 1.8</td>
<td>74.2 ± 14.0</td>
<td>15.9 ± 4.9</td>
<td>5.8 ± 1.5</td>
</tr>
<tr>
<td>20.45 ± 3.7</td>
<td>3.9 ± 0.9</td>
<td>6.2 ± 2.3</td>
<td>16.9 ± 4.4</td>
<td>9.8 ± 1.9</td>
<td>2.08 ± 0.93</td>
<td>133.0 ± 15.0</td>
<td>20.6 ± 2.8</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>2.00 ± 1.0</td>
<td>4.7 ± 1.1</td>
<td>0.6 ± 0.08</td>
<td>1.5 ± 0.06</td>
<td>17.5 ± 2.0</td>
<td>0.08 ± 0.03</td>
<td>201.4 ± 32.0</td>
<td>4.7 ± 1.5</td>
<td>50.6 ± 18.0</td>
</tr>
</tbody>
</table>

$P_{so}$ for mitochondria isolated from cardiac muscle began to decrease as the oxygen concentration declined below about 8 μM (a $P_{so}$ of 0.5–0.7 μM), whereas in cells the region of oxygen dependence began at higher oxygen concentrations, about 20 μM (a $P_{so}$ of 2.3 μM). The difference in the profiles of oxygen utilization was due to two factors: 1) the metabolic state of the mitochondria was substantially different, with the mitochondrial pyridine nucleotides having been more reduced and the energy state having been lower in the suspensions of isolated mitochondria, and 2) in cells, oxygen had to diffuse through a substantial distance from the suspending medium to the mitochondria where oxygen was reduced to water. Thus, for cells but not for mitochondria, there are significant diffusion-induced oxygen concentration differences between the extracellular medium, where it is measured, and the mitochondria. Both the metabolic oxygen dependence and the diffusion-induced oxygen concentration must be considered in order to fully understand the interplay between myocardial oxygenation and cellular energetics.

At concentrations of oxygen greater than 240 μM, the phosphorylation state ratio ([ATP]/[ADP][Pi]) of the quiescent myocytes was high as compared with that found in other types of cells in suspension (for review refer to Ref. 19). The authors are not aware of any reports within the literature that provide values of the phosphorylation potential for cardiac myocytes isolated from mammalian species (creatine concentrations are not normally measured, precluding such a calculation). That the phosphorylation potential is greater than in other cells may be due to the large oxidative capacity of the heart. For comparison, however, in isolated nonjejerating rat heart perfused at 37 °C and electrically paced at 300 beats/min, the creatine phosphate/creatine ratio is about 4-fold less than that observed for the isolated myocytes, 1.2–1.4 versus 6.2 (20, 21). Given the quiescent nature of the isolated cells, it might have been expected that the phosphorylation potential would be higher than that for the perfused heart. Oxygen consumption was, however, comparable for perfused heart and isolated cells (16 versus 13 nmol of O$_2$/g, dry weight-min$^{-1}$, respectively) suggesting that the differences in energetics may in part be attributed to the higher concentrations of oxygen in the latter preparation. The median intracellular oxygen concentration of individual myocytes has been measured in rat subepicardium using myoglobin cryospectroscopy and found to be about 10 μM, varying by less than 3 μM within
clusters of 10–20 myocytes (22). In dog heart, Coburn and co-workers (23), using CO distribution, estimated the mean cellular oxygen concentration to be about 8 μM. These intracellular oxygen concentrations are low enough to be an important factor in determining the [ATP]/[ADP][Pi] value which can be generated by the mitochondria (18). By contrast, the isolated myocytes were exposed to much higher oxygen concentrations than cells in the intact heart, which could give rise to the higher creatine phosphate/creatine ratio in the former.

When the phosphorylation potential of the isolated myocytes was decreased by addition of the uncoupler, FCCP, two responses are relevant and important to the evaluation of the Pso for respiration. In our previous work, using neuroblastoma cells, which were of smaller dimensions than the myocytes, it was shown that: 1) the oxygen pressure difference between the extracellular compartment and the mitochondria contributed little to the measured Pso for respiration and 2) when the cells were treated with uncoupler, the Pso decreased from 1.3 μM to about 0.96 μM despite a severalfold rise in respiratory rate (8). Thus, in the neuroblastoma cells, there was a clear demonstration of the effects of energy metabolism on the oxygen dependence. In myocytes and other large cells, however, the intracellular oxygen gradient can be of greater magnitude. In addition, the number of respiratory proteins and thus the oxidative capacity of cardiac myocytes is much larger per unit volume than for most other cell types (24, 25). When the cardiac myocytes were uncoupled, the response was a 10-fold rise in respiratory rate and a 3-fold increase in Pso. Given that the Pso for respiration for isolated mitochondria (Fig. 1A) and for neuroblastoma cells (8) both decreased upon uncoupling of oxidative phosphorylation, it might have been expected that the Pso for the uncoupled myocytes would also decline. The relative contribution of diffusion to the Pso value of coupled and uncoupled cells is dependent on the rate of respiration, however, and the rise in oxygen consumption resulted in a greater contribution to the diffusion-induced oxygen concentration difference in the myocytes. Amytal, a respiratory chain inhibitor, was therefore added to the media of uncoupled cells to decrease oxygen consumption to that of coupled cells. The data indicate that in resting cardiocytes at 50% of maximal respiratory rate, the diffusion-induced difference in oxygen concentration between the extracellular medium and the mitochondria is approximately 1 μM.

Increasing the concentration of calcium in the media bathing the cells to physiological levels produced a 50% rise in Pso. The change did not result from an enhanced respiratory rate since respiration increased by 10% or less. The addition of calcium did not markedly alter the phosphorylation state ratio of the isolated myocytes, but it did produce a modest reduction in the NAD+/NADH ratio. Calcium has been shown, in vitro, to stimulate the activity of rate-limiting dehydrogenases within the Krebs cycle (26). Koretzky and Balaban (27) have also observed that by enhancing heart rate, thereby diminishing the time for calcium reequilibration, respiratory rate and NADH fluorescence increased. It is noteworthy that the addition of succinate to the substrate mixture (glutamate plus malate) which was provided to the isolated mitochondria resulted in a rise in Pso. We conclude from the results above that the oxygen dependence of respiration is responsive to alterations in the level of substrate availability.

At the half-maximal respiratory rate, the contribution of the oxygen concentration difference between cell exterior and mitochondria was found to be 1.25 μM (Pso of uncoupled myocytes). A major resistance to oxygen flux is offered by the layer of unstirred media surrounding each cell in suspension (28). The oxygen concentration difference across this layer was estimated in our resting cardiocytes to be 0.27 μM, using a Sherwood analogy to estimate the oxygen mass transfer coefficient for the transport of oxygen across the stagnant boundary layer (for details, refer to the Appendix). Possible errors in the estimation of the velocity of the media relative to that of the cells could place this value as high as 0.53 μM or as low as 0.21 μM. The maximum oxygen concentration difference between the plasma membranes and the cell core was calculated to be 1.84 μM for coupled myocytes respiring at the half-maximal respiratory rate (see Appendix). Thus, the decrease in oxygen concentration from the plasma membrane to mitochondria was dispersed throughout the cytosol would range from 0 to 1.84 μM depending on the spatial position of the individual mitochondria. Inclusion of the oxygen concentration drop across the stagnant media layer indicates that mitochondria in situ see an oxygen concentration ranging from 0.27 to 2.11 μM lower than the bulk media. In studies of the oxygen dependence of respiration using isolated cardiac myocytes, Katz et al. (29) and Wittenberg and Wittenberg (30), using monamine oxidase as an oxygen probe or measurements of cytochrome aa3 redox state, respectively, found that the total decrease in oxygen concentration from the bulk phase to the mitochondrion was no greater than 2 torr or about 3 μM. Our findings are consistent with the intracellular oxygen gradients being small relative to the oxygen concentration, about 32 μM, in the capillary lumen of the heart in vivo.

The distribution of oxygen within cells is a matter of some controversy. Two current lines of thought have been put forward: 1) the difference between intracellular and extracellular oxygen concentration is small, less than 3 μM for unstimulated cells (1, 30, 31), and 2) there is a large difference in oxygen concentration (several μM), much of which occurs around the perimeter of mitochondria (32–34). Most of the data in the literature and that presented in the present study are consistent with the former and not the latter view. Using cryomicrospectrophotometry of myoglobin oxygenation, Gayeskii and Honig (31) provided a detailed description of the spatial distribution of oxygen pressure in cross-sections of dog gracilis muscle. Their results indicated that oxygen concentration at the center of the cells was almost equivalent to that at the sarcolemmal membrane. In the Appendix of this report, the intracellular oxygen distribution in isolated cardiac myocytes has been modeled using the data presented in this paper, the measured cell dimensions (radius = 8.5 μM, length of 117 μM), the assumption that mitochondria are homogeneously distributed throughout the cell, and two different values of the oxygen diffusivity coefficient. Based on the work of PaWcott and McNutt (35) who have described the distribution of mitochondria to be strikingly uniform throughout the cells of cat ventricular tissue, it seemed appropriate to assume homogenous distribution of mitochondria in rat cardiac myocytes. The oxygen diffusivity coefficient for the cardiac myocytes was calculated to be 0.3 × 10⁻⁵ cm²/s at room temperature. This value is less than that reported previously for intact tissue. Grote and Thews (36) reported a value of 1.0 × 10⁻⁵ cm²/s for rat heart when measured at 20 °C. Krogh (37) found a value of 0.9 × 10⁻⁵ cm²/s at 20 °C for frog skeletal muscle. The above values are less than 1.5 × 10⁻⁵ cm²/s, considered an average tissue value, which was also used to model intracellular oxygen distribution. The difference between the value for the oxygen diffusivity coefficient found in the present study and those reported previously may be attributed to the experimental systems in which they were determined, i.e. suspensions of isolated cardiac myocytes as
Compared with pieces of tissue, since in the latter, the intercellular matrix would be expected to have an oxygen diffusivity approaching that of saline.

The concentration of oxygen at the core of the resting cardiocytes was nearly the same as that at the sarcolemma when an average tissue oxygen diffusivity coefficient of 1.5 \times 10^{-5} \text{ cm}^2/\text{s} was used to calculate the intracellular oxygen distribution (see Fig. C1 of the Appendix). Using 0.3 \times 10^{-5} \text{ cm}^2/\text{s} as the diffusivity coefficient, the difference in oxygen concentration from the cell membrane to the cell core was predicted to be 1.84 \mu M. If account is taken of these oxygen concentration gradients, it is then possible to extrapolate an intrinsic cellular \( P_{50} \). The analysis showed that the "true" cellular \( P_{50} \) or \( K_m^{o_{so}} \), which accounts for the intracellular oxygen gradients, was about 0.75 \mu M when using 0.3 \times 10^{-5} \text{ cm}^2/\text{s} as the intracellular oxygen diffusivity coefficient and that the \( K_m^{o_{so}} \) rose to 1.7 \mu M based on the intratissue diffusivity of 1.5 \times 10^{-5} \text{ cm}^2/\text{s}. Although 0.75 \mu M is greater than the observed \( P_{50} \) for isolated mitochondria (0.55 \mu M), it is consistent with the experimental data and indicates that the metabolic state of the cells is a major factor in establishing the \( P_{50} \) of the mitochondria in quiescent myocytes. We find that intracellular oxygen gradients are shallow, in agreement with previous investigators (30, 31) but in contrast to the large perimitocondrial oxygen gradient suggested by Jones (32).

Steenbergen and co-workers (38) used NADH surface fluorescence to monitor tissue oxygenation in the saline-perfused rat heart, which showed that discrete heterogeneous concentrations (anoxic) zones were produced by high or low flow hypoxia. Sharp transitions at the border of the fluorescent zones were interpreted as indicating the presence of steep tissue oxygen gradients. The authors suggested that within individual cells mitochondria were either fully aerobic or anaerobic. Our data are not consistent with the existence of such steep oxygen gradients. It appears that the fluorescence pattern observed by Steenbergen and co-workers (38) may be interpreted as anoxic regions due to oxygen gradients along the capillaries, in part arising from the low oxygen-carrying capacity of saline perfusates.

It is generally considered that the role of myoglobin is to facilitate the intracellular diffusion of oxygen and possibly serve as an intracellular buffer or reservoir of oxygen (for an in-depth review on this subject, see Ref. 5). The data from the present study, unfortunately, do not provide definitive evidence for either of these possibilities. The low oxygen concentration differences from plasma membrane to mitochondria supports a facilitative function for oxygen diffusion. On the other hand, the low value calculated for the oxygen diffusivity coefficient might suggest against the latter possibility.

The relationship between the metabolic state, i.e., the phosphorylation state ratio and the redox state of the intramitocondrial pyridine nucleotides, and the oxygen concentration dependence of respiration is a dynamic system rather than a static one. The measured \( P_{50} \) for cytochrome c oxidase for oxygen increases with an increasing mitochondrial energy state, rising from values that are too low to measure accurately in uncoupled mitochondria, <0.05 \mu M, to greater than 0.7 \mu M in coupled quiescent cardiac myocytes. When oxygen delivery to cells is restricted, this deprivation results in a lower energy state and consequent lower \( P_{50} \) for oxygen, allowing the cells to maintain constant rates of respiration and ATP production. Enhanced rates of cycling by the cardiac contractile proteins also decrease the phosphorylation state ratio if the intramitocondrial NAD'/NADH ratio does not change (39). Under these conditions, the apparent affinity of cytochrome c oxidase for oxygen is lowered, allowing the mitochondria to more effectively extract oxygen for sustaining ATP synthesis. Such a mechanism would be especially important when the cardiac myocytes are operating under conditions of near maximal respiratory rate and need to extract as much as possible the oxygen in the blood. Clearly, for heavily working cells in situ, the dominant factors are those that affect the delivery of oxygen to the tissue, i.e. blood oxygenation, coronary flow, and functional capillary surface area (40).

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