Mitochondrial NAD(P)H, ADP, oxidative phosphorylation, and contraction in isolated heart cells

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White, Roy L., and Beatrice A. Wittenberg. Mitochondrial NAD(P)H, ADP, oxidative phosphorylation, and contraction in isolated heart cells. Am J Physiol Heart Circ Physiol 279: H1849–H1857, 2000.—To examine the relationship between mitochondrial NADH (NADHm) and cardiac work output, NADHm and the amplitude and frequency of the contractile response of electrically paced rat heart cells were measured at 25°C. With 5.4 mM glucose plus 2 mM β-hydroxybutyrate, NADHm was reversibly decreased by 23%, and the amplitude of contraction was reversibly decreased by 27% during 4-Hz pacing. With glucose plus 2 mM pyruvate or with 10 mM 2-deoxy-D-glucose, NADHm was maintained during rapid pacing, and the contractile amplitude remained high. Phosphocreatine levels decreased with 2-deoxy-D-glucose administration but not with rapid pacing. Respiration increased to meet the increased ATP demand at 30°C. The data suggest that 1) when NADHm is decreased during rapid pacing with defined substrates, the amplitude of contraction is decreased; 2) the amplitude of contraction during electrical pacing does not change with rate of pacing when both the ATP and NADHm levels are continuously replenished; and 3) the replenishment of NADHm during pacing with physiological substrates may be rate-limited by substrate supply to mitochondrial dehydrogenases. During activation of mitochondrial dehydrogenases, or a significant increase in free ADP induced by 2-deoxy-D-glucose, this rate limitation is bypassed or overcome.

DURING INCREASED CARDIAC OUTPUT the contractile apparatus, together with the pumps and exchangers necessary to maintain transmembrane ionic gradients, create an increased demand for ATP. Recent NMR data in the whole heart have shown that when steady-state ATP utilization is increased, an increased rate of ATP synthesis can be maintained without detectable change in the steady-state levels of high-energy phosphate (7). To maintain constant mitochondrial NADH (NADHm) levels during increased work output when the rate of NADHm oxidation is increased, NADHm must be replenished by increased rates of NADH synthesis. NADH supply is governed by the rates of the mix of mitochondrial dehydrogenases drawing on a common pool of NAD+ and the relative concentrations of the substrates of the mitochondrial dehydrogenases (26).

The beating heart, when perfused with blood containing both glucose and fatty acid, preferentially uses fatty acids as a substrate (12). The heart becomes more dependent on glucose oxidation, however, at high work loads (20).

NADHm is most accurately and reliably measured in isolated heart cells, where oxygen delivery and substrate delivery can be rigorously controlled and the optical path length for fluorescence measurements is relatively constant for any given field of view (24). NADHm in isolated heart cells is decreased by 30% during 4-Hz electrical pacing with glucose and glutamine as substrates (24). To test whether the decrease in NADH is due to a limitation in the rate of supply of reducing equivalents from exogenous substrates, we supplemented the extracellular glucose with fatty acids or the ketoacids acetoacetate or β-hydroxybutyrate. We chose glucose plus β-hydroxybutyrate as our standard reference condition.

We have shown that in the presence of ample oxygen and physiological substrates, the rate of supply of reducing equivalents may limit the steady-state replenishment of NADHm during rapid pacing; the consequent decrease in NADHm is accompanied by decreased contractility. NADHm and the contractile amplitude during pacing may be enhanced by substrate manipulation or by a large increase in free ADP (ADPfree).

MATERIALS AND METHODS

Preparation of Isolated Heart Cells

Heart cells were prepared from adult male rats (350–450 g) by a modification of the procedure reported previously (25). The isolation medium, HEPES-buffered minimal essential medium (MEM), contained (in mM) 117 NaCl, 5.7 KCl, 4.4 NaHCO3, 1.5 NaH2PO4, 1.7 MgCl2, 21.1 HEPES, 10 taurine, 20 glucose, 30 pyruvate, and 2 d- or l-glucose 6-phosphate. The medium was adjusted to pH 7.4 and equilibrated with 95% O2-5% CO2. The heart was cooled to 3°C, perfused in situ with the isolation medium, and cannulated for coronary perfusion. After a 5-minute perfusion period with no-flow conditions, left ventricular myocardium was removed, placed in incremental oxygenated medium (4°C) to remove noncontractile tissue, and equilibrated for 5 minutes. After 10 minutes, the heart was reinserted into a perfusion apparatus equipped with a 1.2-mm i.d. perfusion cannula. The heart was perfused throughout at 3°C for 30 minutes. Left ventricular myocardium was then isolated, equilibrated for 5 minutes, and placed into the perfusion apparatus. Perfusion was maintained for 10 minutes at 3°C, and then temperature was increased to 30°C. After a 5-minute equilibration period, a constant load of 0.5 g was applied to the heart, and end-diastolic pressure was increased by 10 g. After 10 minutes of equilibration, a 5-minute perfusion period was required for substrate manipulation (12). The rate of perfusion was 15–20 ml/min. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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5.4 glucose, and 2L-glutamine and amino acids and vitamins; pH was adjusted to 7.2, and osmolality was adjusted to 292 mosM. Retrograde aortic perfusion of the heart with 0.05% collagenase (Boehringer) was followed by mechanical dissection of the tissue matrix, followed by separation of intact cells with Percoll. The final yield from each heart was $10^{-18}$ cells, of which 80–90% were rectangular and functionally intact (sarcomere length, 2.0 $\mu$m; synchronous contraction with electrical stimulation). Heart cell protein was $4.82 \pm 1.60$ mg protein/106 cells ($n = 25$).

**Measurement of NADHm in Cells**

This method has been described previously (24). The NADHm level is defined as the ratio of NADH fluorescence measured under a defined condition divided by the maximal NADH observed in the presence of 10 $\mu$M rotenone. A Nikon inverted microscope equipped with an epifluorescence attachment and dual, matched photomultiplier tubes was used as a microfluorimeter to record intrinsic fluorescence spectra from myocytes at two wavelengths (415 and 470 nm). Fluorescence emissions from single myocytes (steady-state measurements averaged over 6–30 cells; see Fig. 1, left) or groups of 12–15 myocytes (kinetic measurements; see Figs. 2 and 3) were isolated using a mask with a rectangular opening of constant size in the light path. NADH fluorescence was measured near 470 nm. Cell fluorescence not specific to NADH (background) was measured at 415 nm, a wavelength isoemissive for oxymyoglobin and deoxymyoglobin. This serves as a reference to compensate for differences in cell thickness and light scattering and was subtracted from the reading at 470 nm. Fluorescence measurements were made during a 15-ms, 350-nm illumination of cells. Separate measurement of the NADH of round cells gave the fully oxidized NADH value. The two photomultiplier signals were collected by a Macintosh computer (Apple Computer, Cupertino, CA) connected to a MacLab (CB Sciences, Dover, NH) data acquisition system. Steady-state NADH fluorescence was measured in individual cells at defined periods during the pacing protocol. Cells were superfused with HEPES medium containing 3 mM CaCl2 and equilibrated with 50% O2-45% N2-5% CO2. The control values (shown in Table 1) were the calculated average of initial and recovery values. Occasionally (10–20% of experiments) a continual drop in NADHm was observed among the control, rapid pacing, and recovery. This was attributed to a decline in the viability of the cell during the experimental protocol, and these data were rejected. In the presence of either acetate or iodoacetate, repeated experiments showed that NADH during recovery was
significant lower than that observed initially, even though a significant decrease in NADH was observed during rapid pacing compared with recovery. In these cases the final recovery value was used as the control.

**Pacing Protocol**

Cells were equilibrated for 20 min with stimulation at 0.4 Hz. After 10 min at 4 Hz (comparable to the rate of a normal rat heart) and, finally, to demonstrate reversibility, we then reduced the rate of electrical stimulation to 0.4 Hz for 20 min. Steady-state measurements of single cells are reported in Fig. 1 (left). The continuous time course of NADH fluorescence (shown in Figs. 2 and 3) was obtained by continuous sampling of NADH fluorescence from a field of 12–15 isolated heart cells. Conditions were optimized for survival of the cells, with maximum sustainable contractile amplitude. The pacing protocol could be repeated three or four times without loss of cell viability. Cells (2 × 10^4 total cells) on the microscope stage were superfused with HEPES-buffered MEM (see composition in *Preparation of Isolated Heart Cells*) supplemented with 15 mM NaHCO₃, 3 mM CaCl₂, and 33 nM dobutamine at 25°C. β-Hydroxybutyrate or other additional substrates were given at a dosage of 2 mM when present. The medium was equilibrated with a gas mixture containing 50% O₂–5% CO₂ (balance N₂) to maintain the pH at 7.2 at a temperature of 25°C. Control experiments showed no difference in NADH levels measured with 95% O₂ compared with the 50% O₂ superfusion. Dobutamine was added to enable the cells to follow 4-Hz pacing. Calcium levels were maximized to levels that did not cause calcium overload. Platinum-iridium electrodes at the bottom of the dish delivered bipolar stimulation pulses. Stimulus voltage was increased until cells contracted synchronously (usually <160 V/cm).

**Measurement of Heart Cell Contractions**

The frequency of contraction was controlled by the rate of stimulation. The extent of the contraction was independent of delivered stimulus voltages. Resting cell length was monitored, and if the resting length of the cell changed during recording, the data were discarded. The frequency and amplitude of contraction of individual cardiac myocytes were recorded using a high-speed video camera and video motion edge detector (Crescent Electronics, Orem, UT) in the Nikon microfluorimeter chamber. The video edge detector measured the time between two defined changes in contrast (the ends of the cell, i.e., the time recorded is a measure of cell length) in a scan line of a video image. During pacing, this measurement was recorded continuously and was updated for each field (240 s⁻¹) of video data from the Pulnix camera. Cell length, when plotted as a function of time, provides a measure of diastolic and systolic length, the amplitude of contraction, and the number of contractions. Cells contracted strongly (~20% of the total length of the cell) when stimulated at 4 Hz in control experiments.

**Measurement of Respiration in Cell Suspensions**

The method has been described previously (3–5). Steady-state oxygen consumption rates were determined with a large-diameter, polarographic oxygen electrode (model 2110, Orbisphere, Geneva, Switzerland) in a thermostated brass block held at 30°C. Experiments were carried out under...
The recovery value was taken as the control. *Significantly different (see Ref. 5).

**RESULTS**

We have examined NADH$_{m}$, the contractile amplitude of contraction, high-energy phosphate levels, and the rate of steady-state respiration in heart cells with different substrates and different rates of ATP utilization.

**Effect of Substrates**

**Glucose plus β-hydroxybutyrate.** Mitochondrial NADH. Steady-state measurements of NADH$_{m}$ in single isolated heart cells are shown in Fig. 1 (left). With glucose and β-hydroxybutyrate as substrates, NADH$_{m}$ was significantly and reversibly decreased by ~23% after 10 min of rapid pacing when compared with slow pacing ($P < 0.05$, see Fig. 1A and Table 1). A significant decrease was also observed with glucose supplemented with fatty acids or ketoacids (see Table 1). NADH measurements in the presence of insulin were not significantly different at rest or during pacing (see Table 1). In comparison, Fig. 2A shows a continuous trace of NADH$_{m}$ as a function of time measured in a field containing 12–16 cells. Figure 2 illustrates that a distinct overshoot of NADH occurred during recovery. Such overshoots in response to changes in stimulation rate have been reported previously (2).

**CONTRACTILE RESPONSE.** The effects of electrical pacing of the right ventricular muscle on the rate and magnitude of contraction of single cells are shown in Fig. 1 (right). The cells were able to follow both the slow and fast stimulus rates under all of the conditions explored in these experiments. With glucose plus β-hydroxybutyrate, however, the amplitude of contraction was significantly and reversibly decreased ($28 \pm 2\%$, $n = 7$) at the higher rate of stimulation, whereas diastolic length was maintained.

Table 1. Effects of substrate on contractile amplitude and on NADH$_{m}$ during 4-Hz pacing

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>NADH$_{m}$ ( \text{Control fluorescence} )</th>
<th>NADH$_{m}$ ( \text{Contraction amplitude at 4-Hz pacing (compared with control), %} )</th>
<th>NADH$_{m}$ ( \text{Contraction Amplitude at 4-Hz Pacing (compared with 0.4 Hz), %} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose + β-hydroxybutyrate</td>
<td>0.393 ± 0.03</td>
<td>77 ± 6* (28)</td>
<td>73 ± 2 (7)</td>
</tr>
<tr>
<td>Glucose + octanoate</td>
<td>0.219 ± 0.03</td>
<td>55 ± 6* (18)</td>
<td>73 ± 1 (3)</td>
</tr>
<tr>
<td>Glucose + acetoacetate</td>
<td>0.399 ± 0.04</td>
<td>51 ± 7* (5)</td>
<td>70 ± 5 (3)</td>
</tr>
<tr>
<td>Glucose + acetate</td>
<td>0.344 ± 0.03</td>
<td>25 ± 2* (4)</td>
<td>Not tested</td>
</tr>
<tr>
<td>Glucose + lactate</td>
<td>0.413 ± 0.04</td>
<td>94 ± 8 (27)</td>
<td>91 ± 3 (4)</td>
</tr>
<tr>
<td>Glucose + pyruvate</td>
<td>0.409 ± 0.07</td>
<td>104 ± 5 (13)</td>
<td>96 ± 3 (3)</td>
</tr>
<tr>
<td>Glucose + β-hydroxybutyrate + insulin</td>
<td>0.422 ± 0.06</td>
<td>64 ± 3* (6)</td>
<td>Not tested</td>
</tr>
<tr>
<td>Glucose + β-hydroxybutyrate + iodoacetate</td>
<td>0.303 ± 0.05</td>
<td>60 ± 6* (15)</td>
<td>Not tested</td>
</tr>
<tr>
<td>Glucose + β-hydroxybutyrate + 2-deoxy-D-glucose</td>
<td>0.375 ± 0.04</td>
<td>94 ± 8 (12)</td>
<td>86 ± 4 (3)</td>
</tr>
</tbody>
</table>

All values are means ± SE; nos. in parentheses are no. of cells. Glucose concentration was 5.4 mM; all other substrate concentrations were 2 mM. Iodoacetate concentration when present was 100 μM, insulin when present was 100 μU/ml, and 2-deoxy-D-glucose was 10 mM. Control values of mitochondrial NADH (NADH$_{m}$) are the average of NADH before and after stimulation when these values were not significantly different. With iodoacetate and acetate, NADH$_{m}$ during recovery was always significantly lower than before rapid pacing. Here the final recovery value was taken as the control. *Significantly different ($P < 0.05$) from control.
HIGH-ENERGY PHOSPHATE LEVELS. The phosphocreatine (PCr)-to-ATP (PCr/ATP) ratio measured in resting cells with glucose plus β-hydroxybutyrate in the superfusion chamber at 25°C was 1.85 ± 0.04 and was not significantly decreased during stimulation at 4 Hz (1.71 ± 0.08, n = 20). The PCr/ATP ratio measured in stirred suspensions of cells at 30°C (1.79 ± 0.05, n = 17) was not different from that measured in superfused cells at 25°C. At 30°C, PCr was 50.5 ± 3.5 nmol/mg protein, ATP was 27.4 ± 1.4 nmol/mg protein, and the calculated ADPfree was 51 μM (n = 17) (see Table 2).

STEADY-STATE RATE OF OXYGEN UPTAKE. The cells maintained a steady rate of oxygen consumption in the absence of electrical or chemical stimulation. The steady-state rate of respiration of a stirred suspension of cells at 30°C was 118 ± 0.05 nmol O2/min per 10^6 cells (n = 6) during 4-Hz electrical stimulation (Table 2) (see Fig. 4 for a typical trace). The magnitude of this response did not differ among all substrate regimens tested. The steady-state rate of respiration increased to a value of 118 ± 2 nmol O2/min per 10^6 cells (n = 3) with 3% μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation.

Glucose plus pyruvate or lactate. NADHm and contractile response. In contrast to the results obtained with glucose plus β-hydroxybutyrate, neither NADHm nor the amplitude of contraction decreased significantly during fast pacing with glucose plus pyruvate as a substrate (Fig. 1B). Similar results were observed with glucose plus lactate as a substrate (Table 1).

The effect of pyruvate addition is also demonstrated in the continuous NADHm trace in Fig. 3. NADHm dropped during rapid pacing but did not return to initial values when pacing was subsequently slowed to 0.4 Hz (Fig. 3). This phenomenon was observed in ~20% of the preparations. NADHm increased to a new steady state after the addition of 2 mM pyruvate. A similar effect was observed when lactate was added (data not shown).

HIGH-ENERGY PHOSPHATE LEVELS AND RESPIRATION. With pyruvate plus glucose as a substrate, at 30°C, the PCr/ATP ratio was 1.85 ± 0.08 (n = 6) and ADPfree was 47 μM. With glucose plus lactate, the PCr/ATP ratio was 1.84 ± 0.06 (n = 5) (see Table 2). Respiration of cells in the presence of glucose plus lactate was not
significantly different from that observed with glucose plus β-hydroxybutyrate under comparable conditions (Table 2).

**Effects of Enhanced ATP Utilization and P_i Sequestration with 2-Deoxy-D-Glucose**

The addition of 2-deoxy-D-glucose or iodoacetate inhibited the glycolytic pathway (see Fig. 5). In addition, 2-deoxy-D-glucose caused a substantial increase in ATP utilization for the phosphorylation of a large pool of 2-deoxy-D-glucose.

NADH<sub>m</sub> and contractile response. After a transient overshoot, NADH<sub>m</sub> returned to control values during rapid pacing when cells were superfused with medium containing 10 mM 2-deoxy-D-glucose with glucose plus β-hydroxybutyrate (Figs. 1C and 2A). In contrast, NADH<sub>m</sub> was significantly reduced during rapid pacing when iodoacetate was used to inhibit glycolysis (Table 2). With 2-deoxy-D-glucose, the cells were able to follow rapid rates of stimulation, and the amplitude of contraction at 4 Hz was little reduced compared with that at 0.4 Hz (Fig. 1C and Table 1).

High-energy phosphate levels. A large significant decrease in the PCr/ATP ratio was observed in the presence of 2-deoxy-D-glucose (see Fig. 2B and Table 2). The PCr/ATP ratio in resting cells was decreased to 0.7 in the microfluorimeter chamber at 25°C with 10 mM 2-deoxy-D-glucose compared with 1.85 in the absence of 2-deoxy-D-glucose. The PCr/ATP ratio was 0.8 when the pacing rate was increased to 4 Hz in the presence of 2-deoxy-D-glucose, and again there was no further increase in ADP<sub>free</sub> during rapid pacing. Similarly, in heart cell suspensions stirred at 30°C, the PCr/ATP ratio was significantly decreased from 1.79 to 0.72 ± 0.09 (n = 6) with 2-deoxy-D-glucose, ATP was 26.6 ± 1.4 nmol/mg protein (7.6 mM), and PCr was 19.1 ± 3.0 nmol/mg protein (5.5 mM); the calculated ADP<sub>free</sub> was 216 μM (see Table 2). No significant drop in the PCr/ATP was observed when glycolysis was inhibited with iodoacetate (see Fig. 2B).

Steady-state rate of oxygen uptake. The rate of respiration of unstimulated heart cells was increased to 48 ± 3.7 nmol O<sub>2</sub>/min per 10<sup>6</sup> rectangular cells<sup>−1</sup> (n = 3) in the presence of 2-deoxy-D-glucose, and the rate of respiration during rapid pacing was reversibly and significantly increased to 61 ± 1.3 nmol O<sub>2</sub>/min per 10<sup>6</sup> rectangular cells (n = 3) (see Table 2). An illustrative example is shown in Fig. 4 (top trace). Thus, although initial respiratory rates are higher and ADP<sub>free</sub> was greatly increased, electrical stimulation at 4 Hz with 2-deoxy-D-glucose caused an increase in respiration (61 - 48 = 13 nmol O<sub>2</sub>/min per 10<sup>6</sup> rectangular cells) of the same order of magnitude as that observed in the absence of 2-deoxy-D-glucose (46 - 34 = 12 nmol O<sub>2</sub>/min per 10<sup>6</sup> rectangular cells).

**DISCUSSION**

Mitochondrial NADH

In the heart, NADH fluorescence arises from the mitochondrial compartment (23) and reflects the redox...
state of the free NADH pool of the mitochondrial matrix (10). Cytosolic NADH reflects the lactate-to-pyruvate ratio and is higher in the presence of lactate than of pyruvate (19). Because we find that the fluorescence intensity is not affected by changes in the lactate-to-pyruvate ratio, the fluorescence signal we detect is primarily from NADH$_m$. The PCR/ATP ratio, a sensitive measure of hypoxia, remains high in superfused cells, and oxygen consumption can be increased severalfold over that observed in electrically stimulated cell suspensions by the addition of CCCP, an uncoupler of oxidative phosphorylation, demonstrating that oxygen supply is not limiting.

NADH oxidation. Steady-state oxygen uptake was measured at different states of work output: heart cells at rest, heart cells stimulated at 4 Hz, heart cells uncoupled by CCCP, and heart cells treated with 2-deoxy-D-glucose. During 4-Hz stimulation, the rate of oxygen utilization increased from the resting level of 34 to 46 nmol O$_2$/min per 10$^6$ rectangular cells (see Table 2). The stimulated rate was $\sim$26% of the maximum rate achievable when oxidative phosphorylation was uncoupled with CCCP (118 nmol O$_2$/min per 10$^6$ cells). The magnitude of the steady-state incremental oxygen uptake during 4-Hz stimulation was not affected by inhibition of glycolysis, decreased PCR levels, or the NADH$_m$ level (see Table 2 and Fig. 4). Thus the increase in oxygen consumption and, thereby, NADH oxidation was closely correlated to the rate of stimulation.

Our values of oxygen consumption for resting cardiac myocytes are of the same order of magnitude as those reported earlier (11, 18, 21). The fractional increment in oxygen utilization measured in stirred cell suspensions during 4-Hz pacing reported here is less than that estimated in electrically stimulated single cells on the stage of the microfluorimeter (25). The current data give a more precise measure of the actual increment of oxygen uptake during stimulation, because measurements in the steady-state chamber permit a more accurate measurement of the rate of oxygen uptake (3–5, 21).

The steady-state rate of oxygen uptake gives a direct measure of the rate of oxidative phosphorylation in a tightly coupled system. Assuming a $p/o$ ratio of 2.4 molecules of ATP per oxygen atom (13), the oxygen uptake during 4-Hz stimulation corresponds to a rate of ATP synthesis by oxidative phosphorylation of 215 nmol ATP/min per 10$^6$ rectangular cells. The increase in respiration during 4-Hz pacing is not accompanied by any change in ADP$_{free}$, in agreement with observations in the whole heart (7, 8) and in heart cells (21). These data show that homeostatic mechanisms maintain highly regulated ATP and ADP$_{free}$ levels in the cells during increased activity.

With $\beta$-hydroxybutyrate, lactate, or pyruvate, ADP$_{free}$ was 45–55 $\mu$M (Table 2), not far from the Michaelis-Menten constant of 20–30 $\mu$M for ADP$_{free}$ for oxidative phosphorylation in mitochondria (7). With 2-deoxy-D-glucose, ADP$_{free}$ was increased to 216 $\mu$M, and the rate of oxygen uptake was significantly increased by $\sim$13 nmol O$_2$/min per 10$^6$ rectangular cells in unstimulated heart cells, corresponding to an additional synthesis of 64 nmol ATP/min per 10$^6$ rectangular cells to meet the additional ATP demand. Stimulation at 4 Hz again caused an additional increase in respiration of 13 nmol O$_2$/min per 10$^6$ rectangular cells, corresponding to a total rate of ATP synthesis of 279 nmol ATP/min per 10$^6$ rectangular cells. The increment in respiration with 4-Hz pacing, over and above that observed with 2-deoxy-D-glucose, was similar to that observed during rapid pacing without 2-deoxy-D-glucose. The 2-deoxy-D-glucose model permits us to study the steady state at higher rates of oxidative phosphorylation and at high ADP$_{free}$ levels. 2-Deoxy-D-glucose 6-phosphate, formed by phosphorylation of 2-deoxy-D-glucose, is not further metabolized. ATP synthesis must increase not only to compensate for the loss of glycolytically generated ATP, but also to meet the new demand for ATP to phosphorylate an (essentially) infinite supply of 2-deoxy-D-glucose.

It is noteworthy that PCR levels fall to almost one-third of initial levels when ATP utilization is stimulated by 2-deoxy-D-glucose but not when ATP utilization is increased by electrical pacing (Table 1 and Fig. 2B). Because both oxygen consumption and the frequency of contraction can be increased during pacing, it is unlikely that sequestration of P$_i$ is limiting the rate of ATP synthase activity. The increase in ADP$_{free}$ cannot be attributed to inhibition of glycolysis, because PCR levels are not decreased in heart cells when glycolysis is inhibited by iodoacetate (Fig. 2B). This suggests that with 2-deoxy-D-glucose, ATP synthase activity is not sufficiently rapid to maintain ATP levels at this rate of ATP utilization. In contrast, during electrical pacing in either the absence or presence of 2-deoxy-D-glucose, when intracellular calcium is markedly increased (9), PCR does not drop. Here, ATP synthase activity is increased sufficiently to maintain ATP levels. These results are in accord with suggestions that calcium may directly increase the activity of ATP synthase (22).

NADH regeneration. The rate of delivery of reducing equivalents to NAD$^+$ at the inner mitochondrial membrane is the sum of mitochondrial and cytoplasmic dehydrogenase activities (26) (Fig. 5). To test whether the decrease in NADH observed during pacing is due to a limitation in the rate of supply of reducing equivalents, we supplemented the extracellular substrate supply with fatty acids (acetate or octanoate) or ketoadiacs (acetoacetate or $\beta$-hydroxybutyrate). Substrate transport into the cell is not limited, because there is a high-affinity monocarboxylate transport carrier present (16) and because insulin (which increases glucose entry into the cells) does not affect NADH. With $\beta$-hydroxybutyrate as a substrate, there is an additional NADH$_m$-generating step, $\beta$-hydroxybutyrate dehydrogenase, and NADH levels at both rates of pacing are increased (14, 25). In the presence of a fatty acid substrate, metabolite flux occurs mainly through the $\beta$-oxidation pathway; pyruvate dehydrogenase is inhibited, and glycolytic flux is negligible (1).
Under these conditions, we found that steady-state NADH\textsubscript{m} levels are decreased \textasciitilde50\% during rapid stimulation in the presence of glucose plus fatty acids or ketones (Fig. 1A and Table 1). The data are consistent with the notion that the drop in NADH\textsubscript{m} with glucose and fatty acid substrates during rapid stimulation reflects an insufficient rate of supply of reducing equivalents to NAD\textsuperscript{+} to maintain steady-state NADH\textsubscript{m} levels. The simplest explanation of the data is that during pacing, delivery of reducing equivalents to mitochondrial NADH\textsuperscript{+} is limited by a rate-limiting reaction(s). Possible sites of a rate-limiting reaction could be in glycolytic and the aspartate-malate shuttle pathways, through pyruvate dehydrogenase (when glucose is a substrate), or in the tricarboxylic acid cycle (15, 17).

This limitation in the rate of formation of NADH\textsubscript{m} with abundant oxygen and high ATP levels during pacing is not observed in the presence of lactate, pyruvate, or 2-deoxy-d-glucose. An addition of lactate or pyruvate leads to an increase in the steady-state intracellular concentration of pyruvate, which leads to activation of pyruvate dehydrogenase. The rate-limiting step(s) in the rate of NADH\textsubscript{m} formation during pacing is then overcome or bypassed. The rate-limiting step is also overcome or bypassed when ADP\textsubscript{free} is substantially increased by 2-deoxy-d-glucose.

Contractile Response During Pacing

The most sensitive index of contractile dysfunction in these experiments is the amplitude of contraction of single cells in response to rapid electrical pacing. During rapid pacing with glucose plus fatty acids, the amplitude of contraction was significantly and reversibly decreased (Fig. 1A and Table 1) when NADH\textsubscript{m} was decreased, even though the rate of oxygen uptake was significantly increased (Table 2) and the PCr/ATP ratios were not detectably affected. The effective work output is diminished even though the rate of oxygen consumption at comparable ADP\textsubscript{free} does not differ detectably from that when NADH\textsubscript{m} levels are high. This may reflect a decreased rate of ATP synthesis per mole of O\textsubscript{2} consumed, as a consequence of reduced mitochondrial membrane proton electrochemical potential (\(\Delta \mu \text{H}^+\)) when NADH levels fall, because the \(\Delta \mu \text{H}^+\) and NADH are in near equilibrium (6).

Contractile amplitude has been shown to decrease with either decreased intracellular pH or with decreased systolic calcium as a result of direct effects on the contractile mechanism. However, during stimulation intracellular pH does not decrease significantly (24), and intracellular calcium is significantly increased. A decrease in the strength of contraction is not observed when NADH\textsubscript{m} is maintained during pacing, either by the enhanced activation of pyruvate dehydrogenase or by a marked increase in ADP\textsubscript{free} during 2-deoxy-d-glucose treatment. Thus, when ATP levels and NADH\textsubscript{m} are nearly constant, the rate of oxidative phosphorylation and work output can be increased by rapid pacing, even at significantly decreased PCr levels, as long as oxygen supply is not limiting.

The following conclusions can be stated in summary: 1) During 4-Hz pacing, the rate of oxygen uptake of isolated heart cells is increased to meet increased ATP demand without a change in steady-state high-energy phosphate levels. The amplitude of contraction during rapid electrical pacing does not become limited as long as NADH\textsubscript{m} levels are maintained. 2) With physiological substrates, NADH\textsubscript{m} and the contractile amplitude of contraction are both decreased during pacing. We suggest that the rate of regeneration of NADH\textsubscript{m} during rapid pacing is insufficient to replenish the steady-state levels of NADH\textsubscript{m}. With glucose and fatty acid substrates, a rate-limiting reaction(s) in the glycolytic cycle, the aspartate-malate shuttle, pyruvate dehydrogenase, the tricarboxylic acid cycle, and/or \(\beta\)-oxidation may limit the supply of carbon substrates to mitochondrial dehydrogenases. 3) The addition of either of the substrates that activate pyruvate dehydrogenase or 2-deoxy-d-glucose, which increases ADP\textsubscript{free} fourfold, overrides the rate limitation and maintains both NADH\textsubscript{m} and the amplitude of contraction during pacing. 4) When NADH falls during pacing, the increment in the oxygen consumption rate is not detectably different from that observed when NADH levels are maintained during pacing, but the effective work output is decreased, suggesting that the efficiency of oxygen utilization for contractile work is diminished. This may reflect a decreased rate of ATP synthesis per mole of O\textsubscript{2} consumed, as a consequence of a reduced \(\Delta \mu \text{H}^+\), when NADH levels fall. 5) The rate of ATP synthesis by ATP synthase is enhanced to meet the ATP demands of 4-Hz stimulation without a change in high-energy phosphate levels. With 2-deoxy-d-glucose, however, a metabolic ATP demand of similar magnitude results in a large decrease in PCr. Because the PCr drop was not observed when glycolysis was inhibited by iodoacetate, the data are in accord with the suggestion that increased intracellular calcium levels during electrical pacing may directly enhance the activity of ATP synthase.

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