Effects of Calcium on Mitochondrial NAD(P)H in Paced Rat Ventricular Myocytes

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ABSTRACT The response of the steady-state level of mitochondrial NAD(P)H of individual cardiac myocytes to substrate and to pharmacological alteration of intracellular calcium was investigated using a defined pacing protocol. Rapid pacing (5 Hz) reversibly decreased the NAD(P)H level and increased oxygen consumption whereas phosphocreatine and ATP levels did not change significantly. Verapamil plus $NiCl_2$ blockade of calcium channels abolished contractions. Ryanodine, which prevents calcium-induced calcium release, also stopped cell contraction. NAD(P)H levels do not change in the absence of contraction. Blockade of sarcolemmal K^+ channels did not stop contraction, and NAD(P)H levels reversibly decreased during rapid pacing. Thus rapid contractions are associated with a reversible decrease in NAD(P)H levels. Ruthenium red blockade of Ca^{2+} entry into mitochondria did not block contraction but significantly decreased NAD(P)H levels in both slowly paced (0.5 Hz) and rapidly paced cells. The simplest explanation of these data is that the steady-state reduction of NAD(P)H is strongly dependent on the rate of ATP utilization and not on sarcoplasmic Ca^{2+} levels when the oxygen and substrate supplies are not limiting and the intracellular calcium regulation is maintained. An effect of intracellular Ca^{2+} on NAD(P)H is observed only when Ca^{2+} entry into mitochondria is blocked with ruthenium red.

INTRODUCTION

The rate of oxidative phosphorylation by cardiac mitochondria must keep pace with changing rates of ATP utilization imposed by changing work output. Many parameters of mitochondrial function may adjust to achieve this change. Here we focus attention on the steady-state extent of reduction of mitochondrial NAD(P)H/NAD(P). Reducing equivalents from many substrate dehydrogenation reactions enter the mitochondrial electron transport chain through NAD(P)H/NAD(P). This large pool (~1–2 mM) is in near equilibrium with reactions of many mitochondrial chain enzymes (Ugurbil and From, 1993), and its state of reduction depends on the balance between the rates of oxygen supply, substrate supply, and ATP utilization. When the delivery of oxygen to cytochrome oxidase is not limiting, the steady-state level of reduction of NAD(P) to NAD(P)H reflects the balance between the rate of inflow of reducing equivalents from substrate and the rate of dissipation of the electrochemical gradient across the mitochondrial inner membrane ($\Delta\mu$) by the action of mitochondrial ATP synthase.

In the heart, voltage-dependent sarcolemmal calcium channels open with each depolarization, permitting calcium to enter the cell and activate a large spike of calcium release from the sarcoplasmic reticulum, relieving troponin inhibition and initiating contraction and ATP utilization by actomyosin ATPase (Hille, 1984). Calcium uptake by the sarcoplasmic reticulum and Na/Ca exchange across the sarcolemma return sarcoplasmic calcium to diastolic levels.

Mitochondrial reactions catalyzed by pyruvate (PDH), α-ketoglutarate, and isocitrate (ICDH) dehydrogenases may be activated by increased mitochondrial Ca^{2+}. Both (mean) cytosolic and mitochondrial Ca^{2+} increase in cardiac myocytes when cells are paced rapidly in the presence of β-adrenergic agonists. Under these conditions, the active form of PDH is reported to increase (Di Lisa et al., 1993). However, in earlier experiments using glucose as the sole substrate (White and Wittenberg, 1993), steady-state levels of NAD(P)H actually decreased during pacing. Thus activation of mitochondrial dehydrogenase activity during pacing enhances the rate of substrate dehydrogenation but does not increase the steady-state level of mitochondrial NAD(P)H.

Harris and Das (1991) report that the capacity of ATPase/synthase is increased in a calcium-dependent manner during stimulation of cultured cardiac myocytes, and Scholz and Balaban (1994) report that F_1-ATPase activity is increased by 30% in whole heart beating in the presence of β-adrenergic agonists. The suggestion is that increased sarcoplasmic calcium during pacing will activate ATP synthase capacity, thus permitting a higher maximal rate of ATP synthesis during increased demand. A higher ATP synthase rate will increase the rate of dissipation of the mitochondrial electrochemical gradient ($\Delta\mu$), and a decrease in $\Delta\mu$ will be reflected in a decrease in NAD(P)H as a consequence of the interconnection of these two substrates by near-equilibrium reactions. This suggested that increased mitochondrial calcium during pacing might increase mitochondrial NAD(P)H if the dehydrogenase activation by Ca^{2+} dominates and might decrease mitochondrial NAD(P)H if the ATPase/synthase activation dominates. No change in NAD(P)H

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would indicate a balance achieved by a matched calcium-dependent stimulation of both reactions (Harris and Das, 1991).

In this report we use single cells freshly isolated from adult rat heart to test the effects of modulation of intracellular calcium on steady-state levels of NAD(P)H using a standardized protocol where oxygen and substrate availability are made not limiting and ATP utilization is increased reversibly. Ruthenium red is used to inhibit calcium transport into mitochondria (Moore, 1971; Gunther and Pfeiffer, 1990), thus preventing calcium-dependent activation of both mitochondrial dehydrogenases and ATPase/synthase. The simplest explanation of the findings of the present experiments is that the steady-state reduction of mitochondrial NAD(P)H during pacing is strongly dependent on the rate of ATP utilization at the myofilaments and not on sarcoplasmic Ca^{2+} levels. At the unphysiologically low mitochondrial calcium levels achieved with ruthenium red, the effect of calcium to stimulate mitochondrial dehydrogenases dominates the level of NAD(P)H. An effect of intracellular Ca^{2+} on mitochondrial NAD(P)H levels is observed only when Ca^{2+} entry into mitochondria is blocked with ruthenium red.

MATERIALS AND METHODS

Ruthenium red was obtained from Sigma (St. Louis, MO). The purity was 15.3% on the basis of absorbance spectra using an extinction of 792 at 532 nm for 1% ruthenium red (Luft, 1971). The concentration used here, 6 μg/ml of 15.3% ruthenium red, is equivalent to 1.2 μM of 100% ruthenium red. All other reagents were purchased from Sigma unless specified otherwise.

Dissociation of the heart into cells

Heart cells were prepared by a modification of the procedure of White and Wittenberg (1993). Retrograde aortic perfusion of the heart was begun immediately after removal with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH = 7.5)-buffered minimal essential medium (MEM) containing 117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM $NaH2PO_4$, 1.7 mM $MgCl_2$, 21.1 mM HEPES, 5.4 mM glucose, amino acids and vitamins, 2 mM L-glutamine, and 10 mM taurine; the pH was adjusted to 7.2 with NaOH. All solutions were prepared with American Society of Tests and Materials type I water produced by treating house-distilled water with a commercial mixed-bed ion exchanger followed by a charcoal filter and finally a Millipore filter. This solution was 292 mOsm, and the free calcium activity was 2–5 μM, as measured with a Möller (Zurich, Switzerland) calcium ion-selective electrode. For the perfusion steps, we added 10 μM $CaCl_2$ to MEM to give a final calcium activity of 7 μM. After blood washout, the perfusion medium was supplemented with 0.045% collagenase (Boehringer Mannheim type B; batches are selected for a high yield of viable myocytes). This solution was recirculated at 7 ml/min for 25 min. All perfusion solutions were maintained at 32°C and equilibrated with a water-saturated 85% O_2/15% N_2 gas mixture. The heart was removed from the perfusion apparatus and cut into 8–10 pieces in 10 ml incubation medium containing 0.09% collagenase. The composition of incubation medium was the same as MEM (above), with the addition of 1.0 mM $CaCl_2$ and 0.5% bovine serum albumin (BSA, Fraction V). The suspension was gently swirled in 50 ml Erlenmeyer flasks at 32°C by a wrist-action shaker. Cells were decanted from the tissue, washed by low-speed centrifugation (34 × *g*) to complete removal of collagenase and some subcellular debris, and suspended in incubation medium. Incubation of the tissue suspension with collagenase was repeated at least two more times. After being washed, the cells were purified with Percoll (Pharmacia Fine Chemicals, Uppsala Sweden). Cells were suspended in isotonic Percoll diluted with incubation medium to a final concentration of 0.2–0.4 × 10^6 cells/ml and 41% Percoll. Ten-milliliter portions of the Percoll-cell suspension in 15 ml Corex centrifuge tubes were centrifuged at room temperature for 10 min at 34 × *g*. Intact rectangular cells sedimented to the bottom of the tube, and rounded cells formed a distinct layer at the surface. The pellet of intact cells was washed to remove Percoll, suspended in incubation medium, and maintained at 34°C. The yield was 1.5 ± 0.7 × 10^7 cells per heart; 89.5 ± 0.8% (n = 12 hearts) of the ventricular myocytes were rectangular. Sarcomere lengths were 1.98 ± 0.12 μm (n = 10 cells). Cells contracted and relengthened specifically in response to electrical stimulation (15 V/mm, 6 ms duration) and were quiescent otherwise.

Measurements of mitochondrial NADH with a microfluorimeter

The mitochondrial NAD(P)H level is defined as the ratio of NADH fluorescence measured at a particular condition divided by the maximal NADH observed in the presence of 10 μM rotenone at 23°C.

The measurement of mitochondrial NAD(P)H of single cells by fluorescence was described previously (White and Wittenberg, 1993). Briefly, an inverted microscope (Nikon Diaphot, Garden City, NY) equipped with an epifluorescence attachment and dual photomultiplier tubes was used as a microfluorimeter to record intrinsic fluorescence spectra from single myocytes at two wavelengths (415 and 470 nm). Fluorescence emission from a single myocyte was isolated using a mask with a rectangular opening of constant size in the light path. The currents from the two photomultipliers were converted to voltages, filtered, and then digitized (256 samples in 50 ms, 12-bit resolution). Optimal resolution of NADH was observed at 470 nm and recorded by photomultiplier 1. Intrinsic cell fluorescence (cell background) was measured at 415 nm, a wavelength isoemissive for oxymyoglobin and deoxymyoglobin. As recorded by photomultiplier 2, this value served as a reference to compensate for motion artifacts, differences in cell thickness, and light scattering. The duration of illumination, the sampling rate, and the number of samples digitized were selected to avoid bleaching of the NADH fluorescence and to average noise. The mean difference (256 samples) between the signals from photomultipliers 1 and 2 was used to calculate NADH. We previously presented a calibration curve showing that this method gives a linear relationship with standard solutions of NADH/NAD mixtures (White and Wittenberg, 1993). The background fluorescence from the medium was recorded at 470 and 415 nm in the absence of a cell image in the observation area and was subtracted from the cell image signal from each photomultiplier. Maximum NADH fluorescence was recorded from 10 μM rotenone-treated cells. Minimum NADH fluorescence (usually near zero) was recorded from single "rounded" cells.

Cells (1 × 10^4 total cells) in the perfusion chamber on the microscope stage were superfused with MEM supplemented with 9.5 mM $NaHCO_3$ (total 15 mM), 1 mM $CaCl_2$ (total 2 mM), 0.05 μM arterenol, and 2 mM β-hydroxybutyrate (β-OH butyrate) (except the experiment described in Fig. 1) at 23°C. The superfusion medium was equilibrated with 95% O_2/5% CO_2 for the duration of the experiment. Platinum-iridium electrodes at the bottom of the chamber delivered bipolar stimulation pulses. Cells were equilibrated for 20 min at the pacing rate indicated before the initial NAD(P)H measurement was recorded from six identifiable cells between the stimulating electrodes. The selected cells were followed individually throughout each experiment. The stimulation rate was increased to 5 Hz (300 beats/min), and NAD(P)H in the same six cells was measured during pacing after 10 min. To demonstrate reversibility, the rate of electrical stimulation was reduced to the initial rate for 20 min, and a final measurement of NAD(P)H was recorded from each cell.

Oxygen consumption measurements

Oxygen partial pressure (P_{O2}) was measured with a small (0.125-inch body diameter) polarographic membrane-covered oxygen-sensing electrode. The electrode (Instech Corp., Plymouth Meeting, PA) was placed near the bottom of the observation chamber between the stimulating electrodes in the downfield path of the superfusate flow. The oxygen electrode was calibrated with superfusion medium gas-equilibrated with 95% oxygen/5% CO_2, 95% air/5% CO_2, and 95% nitrogen/5% CO_2 gas mixtures. The response was linear throughout this range. Electrical pulses from the stimulator did not affect oxygen electrode readings in the absence of cells. Superfusion medium equilibrated with 95% O_2/5% CO_2 was passed through the chamber at a flow rate of 1.7 ml/min. Cells (1.25×10^5) were added to the chamber and allowed to settle, and superfusion was restarted with medium equilibrated with 95% oxygen/5% CO_2. P_{O2} was recorded at steady state: before the cells were added (p_1), after equilibration of the cells in the absence of stimulation (p_2), and during stimulation at 0.5 Hz (p_3) and 5.0 Hz (p_4). Stimulation was then decreased to 0.5 Hz and finally stopped to repeat measurements p_3 and p_2.

The steady-state P_{O2} in the dish, in the presence of cells, reflects the oxygen supplied by the superfusing medium minus diffusive loss of oxygen from the dish minus cellular uptake of oxygen by the cells. Therefore the difference in P_{O2} with no cells in the dish minus P_{O2} in the presence of cells reflects the cellular respiratory oxygen uptake. The difference in steady-state P_{O2} measured at each pacing rate is normalized to the P_{O2} difference (p_1 minus p_2) observed with cells at rest. This normalized value is a measure of the ratio of cell respiration during pacing relative to resting respiration. Thus $p_1 - p_3/p_1 - p_2$ = normalized respiratory oxygen consumption of cells paced at 0.5 Hz. $p_1 - p_4/p_1 - p_2$ = normalized respiratory oxygen consumption of cells paced at 5 Hz.

Measurements of phosphocreatine (PCr)/ATP ratio

For analyses of populations of resting cells, portions of the heart cell suspension ($\geq 1.5 \times 10^6$ cells) were deproteinized with ice-cold perchloric acid. In order to compare PCr/ATP ratios in stimulated heart cells with unstimulated controls, 0.45×10^6 cells were superfused in the microscope perfusion chamber, as described above. Control cells were perfused for 30 min without stimulation and then removed for deproteinization and high-pressure liquid chromatography analysis. Stimulated cells were perfused for 20 min without stimulation, then stimulated for 10 min at 5 Hz, and removed for deproteinization and high-pressure liquid chromatography analysis. A 0.1-ml portion of the neutralized deproteinized extract was filtered and analyzed by high-pressure liquid chromatography. ATP and PCr were separated on an anion-exchange column (Whatman (Hillsboro, OR) Partisil SAX 10, 15-cm long) using 5 mM NH_4PO_4, pH 2.85, for 10 min at 1 ml/min, followed by a linear gradient to 750 mM NH_4PO_4, pH 4.4, for 25 min at 2 ml/min. PCr is eluted near 18 min, and ATP is eluted near 30 min.

Statistical analysis

Results are presented as mean ± SE. The null hypothesis for data obtained with treatments on different hearts was tested using Student's *t*-test (unpaired). The null hypothesis for data obtained in measurements of the same cells during different rates of pacing was determined using the paired *t*-test. The paired *t*-test was also used to determine the statistical significance of different interventions carried out on cells from the same heart preparation. Data are considered significantly different when $p \leq 0.05$.

RESULTS

In an earlier study (White and Wittenberg, 1993), we used 5.4 mM glucose and 2 mM glutamine as the sole exogenous substrates. We now find that the addition of 2 mM β-OH butyrate substantially increases the NADH and PCr levels in the cells. The ratio of PCr to ATP is increased significantly from 1.77 ± 0.09 to 1.95 ± 0.08 ($p < 0.0001$; $n = 25$ hearts). The fractional reduction of mitochondrial NAD(P)H in resting cells increased significantly from 0.22 ± 0.03 to 0.41 ± 0.03 ($p < 0.0001$; $n = 23$ for glucose, $n = 28$ for β-OH butyrate plus glucose; Fig. 1). With either substrate regimen, mitochondrial NAD(P)H was significantly and reversibly decreased ~30% from the resting state by electrical stimulation at 5 Hz ($p \leq 0.0008$). The quantitative change in mitochondrial NAD(P)H between the resting and stimulated states was 0.08 in the absence of β-OH butyrate and 0.10 in its presence. These differences are are not significantly different ($p = 0.53$). The extent of

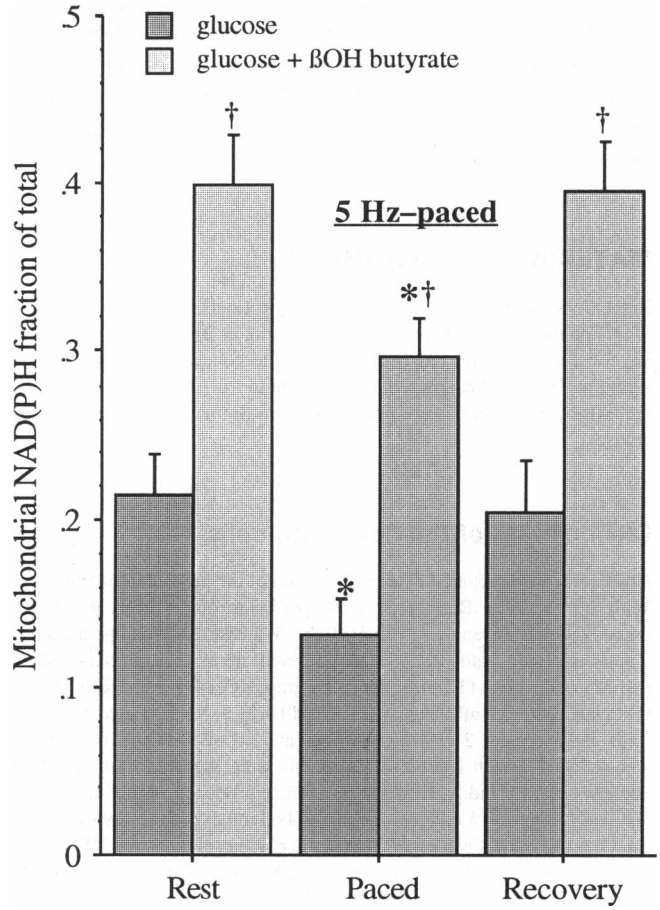

FIGURE 1 The effects of extracellular substrate on mitochondrial NAD(P)H of single cardiac myocytes. Cells were superfused with medium equilibrated with 95% O_2/5% CO_2. Cells were stimulated at 5 Hz after an initial rest period with no stimulation. Stimulation was turned off for the 20-min recovery. Supplementing the medium with β-OH butyrate strongly increased mitochondrial NAD(P)H of cells at rest ($p \leq 0.0001$), during pacing ($p \leq .00041$), and after recovery ($p \leq 0.0020$). With both substrates, NAD(P)H is significantly and reversibly decreased during pacing ($p \leq 0.0006$, $n = 29$ in the presence of glucose alone; $p \leq 0.0008$, $n = 23$ in the presence of both glucose and β-OH butyrate). * = significantly different from the rest, within treatment groups. † = significantly different from glucose alone, with the same rate of stimulation. Data are mean ± SE.

reduction of NAD(P) was not significantly different during the initial rest and final recovery periods, indicating that the effect of contraction was reversible.

To more closely approximate the situation in the beating heart, we carried out all subsequent experiments in glucose medium supplemented with 2 mM β-OH butyrate. We also maintained slow-paced contractions at 0.5 Hz (30 beats/min) during the initial and recovery phases. Mitochondrial NAD(P)H levels were the same as those obtained with no pacing (compare light gray bars in Figs. 1 and 2). Our new pacing protocol is a 20-min period of 0.5 Hz pacing followed by 10 min of rapid pacing at 5 Hz followed by a recovery phase of 20 min at 0.5 Hz. Mitochondrial NAD(P)H levels were not significantly different with this new pacing protocol ($n = 26$) compared with the resting to rapid stimulation protocol used previously ($n = 18$).

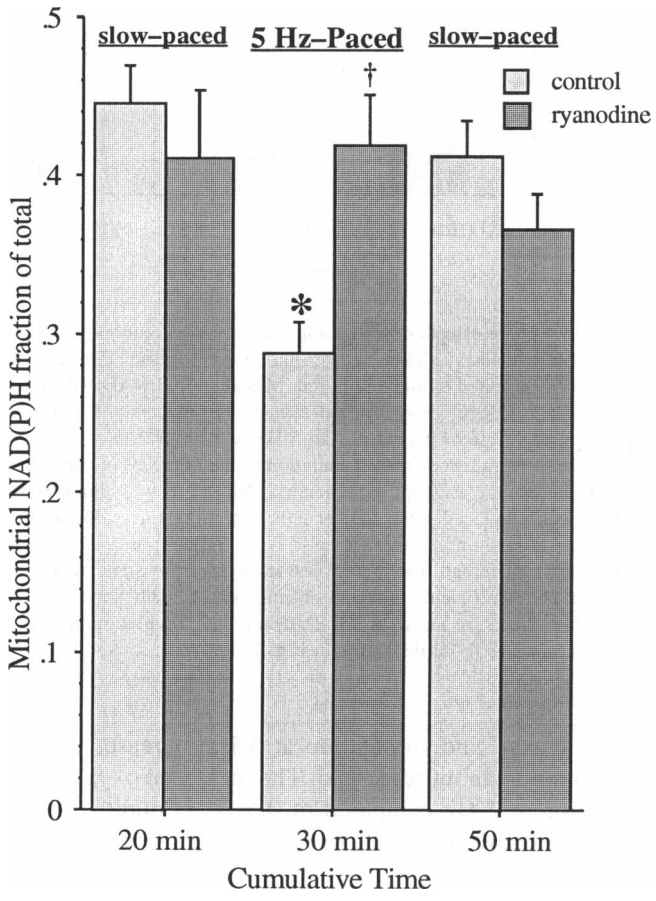

FIGURE 2 The effect of ryanodine on mitochondrial NAD(P)H of single cardiac myocytes. Ryanodine blocks the sarcoplasmic release channel and thus calcium-induced calcium release. Superfusion of isolated myocytes with 5 μM ryanodine stops contraction. Initially cells were slowly stimulated at 0.5 Hz for 20 min. Stimulation was increased to 5 Hz during the next 10 min, and then stimulation was reduced to 0.5 Hz for 20 min. Mitochondrial NAD(P)H does not change significantly when stimulation is increased from 0.5 to 5 Hz ($n = 14$), in contrast to control (*light bars*). * = significantly different from initial value, within the same treatment group. † = significantly different from control, with same rate of stimulation. Data are mean ± SE.

Fig. 2 shows the effects of 5 μM ryanodine on isolated heart cells. Ryanodine is known to block the sarcoplasmic reticulum calcium-release channel at this concentration and thus calcium-induced calcium release. We find that in the presence of ryanodine, cells do not contract in response to electrical stimulation. Mitochondrial NAD(P)H is not changed during rapid electrical pacing (in contrast to control). PCr/ATP was not significantly altered by the addition of ryanodine ($p > 0.05$; $n = 4$ hearts).

The blocking of sarcolemmal calcium channels with verapamil (10 μM) plus $NiCl_2$ (50 μM) also stopped contractions in response to electrical stimulation. Mitochondrial NAD(P)H did not decrease in response to increased electrical pacing. There was no significant difference in mitochondrial NAD(P)H before and after 5 Hz pacing; the response to pacing was completely reversible (data not shown). PCr/ATP was not significantly altered by the addition of verapamil plus $NiCl_2$ ($p > 0.05$; n = three hearts). These results are entirely similar to those with ryanodine.

Fig. 3 shows the effects of blocking sarcolemmal K^+ channels with 5 mM tetraethylammonium bromide (TEA) plus 50 μM 4-aminopyridine (4-AMP). In contrast to ryanodine or verapamil plus NiCl, the addition of K^+ channel blockers did not stop contractions or the reversible decrease in mitochondrial NAD(P)H in response to increased pacing. Cells became shortened toward the end of the 5-Hz pacing; contractions were incomplete. Although mitochondrial NAD(P)H increased during "recovery," it did not return to initial values in the 20-min time period allocated. PCr/ATP was not different in resting cells in the absence or presence of TEA plus 4-AMP (n = three hearts).

Mitochondrial NAD(P)H decreased reversibly in response to pacing at 5 Hz in cells treated with 6 μg/ml ruthenium red (Fig. 4). Unlike control cells, mitochondrial NAD(P)H did not fully return to control values during recovery. It is notable that mitochondrial NAD(P)H is significantly decreased from control values in the presence of 6 μg/ml ruthenium red during both the slow-paced and the rapidly paced phases of the protocol. PCr/ATP was not significantly changed by the addition of 6 μg/ml ruthenium red to resting heart cells ($p > 0.05$; $n = 15$ hearts). Table 1 shows that during 5 Hz stimulation in these well oxygenated cells, the PCr/ATP level dropped slightly but not significantly in both the presence and the absence of ruthenium red.

Ruthenium red is known to inhibit calcium entry into mitochondria (Gunther and Pfeiffer, 1990). To confirm the appropriateness of the dose, mitochondria were prepared by a modification of the method of Berkich et al. (1991). Mitochondria suspended in solutions containing 1 mM $CaCl_2$ become uncoupled (maximally activated respiration in the absence of added ADP as measured by polarographic O_2 uptake). Ruthenium red (0.1 μg/ml) partially blocks and 0.5 μg/ml ruthenium red completely blocks calcium-induced uncoupling. This shows that 0.5 μg/ml ruthenium red is sufficient to block calcium entry into isolated mitochon-

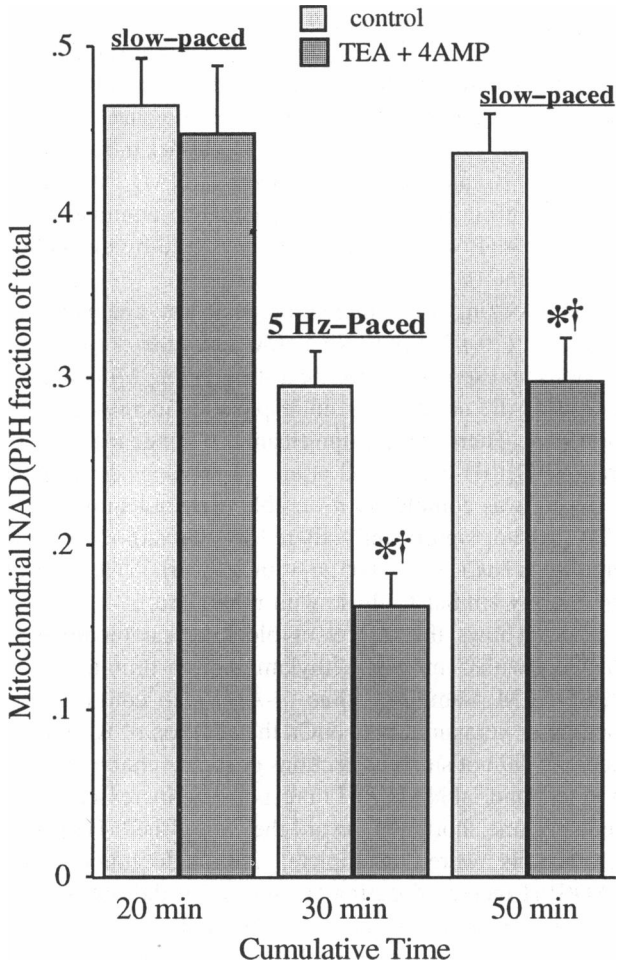

FIGURE 3 The effects of potassium channel blockers on mitochondrial NAD(P)H of single cardiac myocytes. Heart cells treated with 5 mM tetraethylammonium bromide (TEA) plus 50 μM 4-AMP, agents that block sarcolemmal K^+ channels, continue to contract in response to pacing. Sarcoplasmic Ca^{2+} will increase as a consequence of the prolonged cardiac action potential induced by these agents. Mitochondrial NAD(P)H in cells treated with TEA plus 4-AMP is significantly decreased during pacing ($p \leq 0.0084$; $n = 6$), with partial (incomplete) return to initial levels after 20-min recovery. * = significantly different from initial levels, within treatment groups. † = significantly different from control cells, at the same rate of stimulation. Data are mean ± SE. Perfusion and stimulation protocols were the same as those described in Fig. 2.

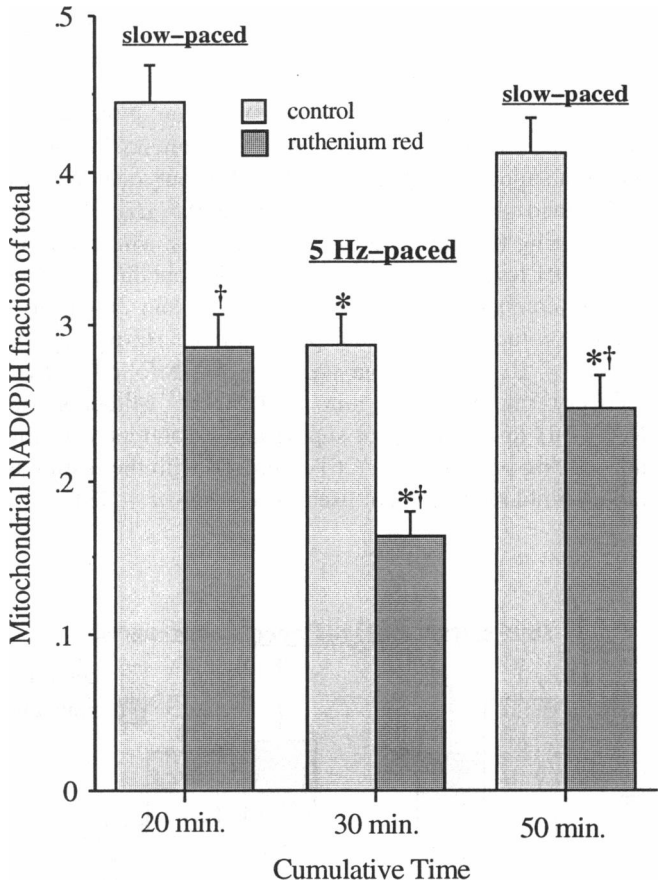

FIGURE 4 The effects of ruthenium red on mitochondrial NADH in single heart cells. Calcium entry into mitochondria through the uniporter is inhibited by ruthenium red. Mitochondrial NAD(P)H in the presence of 6 μg/ml of ruthenium red was significantly lower than control at each rate of pacing (0.5 to 5 Hz; $p \leq 0.0003$; $n = 16$). The cells contracted in response to stimulation in the presence of ruthenium red. During pacing at 5 Hz, mitochondrial NAD(P)H was significantly and reversibly reduced in the control cells ($p \leq 0.0001$; $n = 35$), as well as in the ruthenium red-treated cells ($p \leq 0.0001$; $n = 16$). * = significantly different from initial value, within the same treatment group. † = significantly different from control, with same rate of stimulation. Data are mean ± SE. Perfusion and stimulation protocols were the same as those described in Fig. 2.

dria and confirms that the dose of 6 μg/ml used in our heart cell experiments is reasonable.

Fig. 5 shows the effect of pacing at 0.5 and 5 Hz on the oxygen consumption of heart cells in the microfluorimeter chamber. Each oxygen uptake measurement is normalized to the resting oxygen uptake measured before and after stimulation in the same experiment. The P_{O2} of the medium drops rapidly to a new steady state during pacing with 0.5 Hz and drops again to a new steady state during 5 Hz pacing. The P_{O2} of the medium was never below 20 mm Hg even during 5 Hz stimulation. Note that stimulation at 0.5 Hz in the presence of 50 nM norepinephrine increases oxygen uptake about threefold compared to rest, and stimulation at 5 Hz increases oxygen consumption about sevenfold. The increased oxygen consumption at 5 Hz compared to 0.5 Hz is highly significant, in both control cells ($n = 4$) and ruthenium-treated cells ($n = 2$) ($p < 0.005$ and $p < 0.02$, respectively). The normalized oxygen consumption is not significantly dif-

TABLE 1 PCr/ATP levels during 5 Hz stimulation

Substrate	PCr/ATP at rest	PCr/ATP during stimulation
Glucose + β-OH butyrate	1.76 ± 0.08	1.68 ± 0.12 ($n = 10$)
Glucose + β-OH butyrate + ruthenium red	1.71 ± 0.08	1.64 ± 0.06 ($n = 6$)

These data are not significantly different, $p > 0.05$.

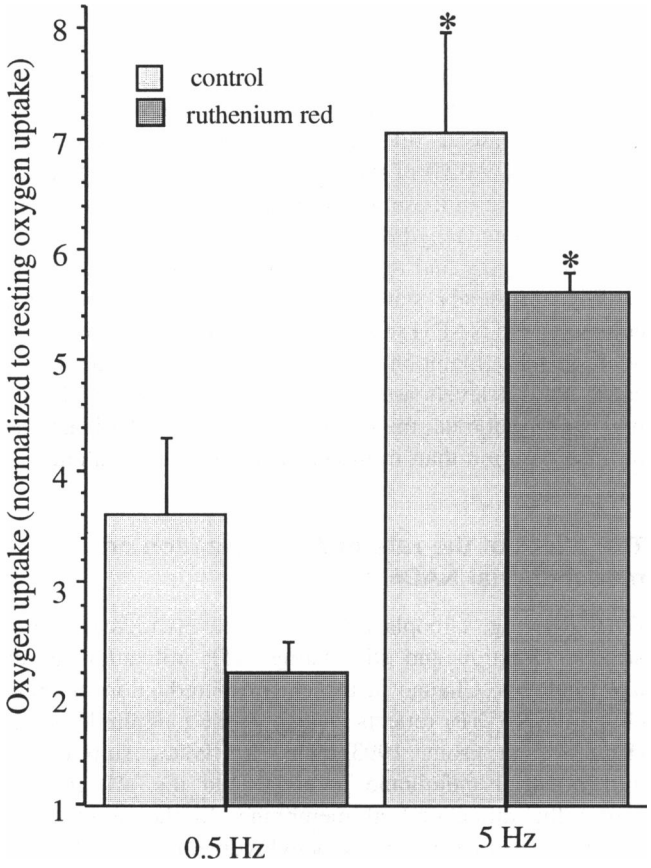

FIGURE 5 The effects of electrical pacing on the oxygen consumption of isolated cardiac myocytes. Oxygen electrode measurements were recorded in the absence of stimulation before and after the pacing protocol. This value reflects the resting O_2 uptake. The cells were stimulated at 0.5 Hz for 5–10 min until steady-state oxygen measurement was achieved, and the pacing was then increased to 5 Hz. Each oxygen uptake measurement is normalized to the resting oxygen uptake of the same population (125,000) of cells before and after stimulation. A significant threefold increase in the normalized oxygen consumption is observed during 0.5 Hz stimulation in the control cells ($n = 4$). When pacing is increased to 5 Hz, the relative oxygen consumption is significantly increased over that observed at 0.5 Hz. There is no significant difference in the normalized oxygen consumption measured at each rate of stimulation between control cells and ruthenium red-treated cells. * = significantly different from 0.5 Hz-paced cells, within treatment groups.

ferent in control cells compared to ruthenium-treated cells at either 0.5 Hz or 5 Hz pacing.

DISCUSSION

Electrical pacing of single cardiac myocytes increases work output, and the rate of oxidative phosphorylation is increased to maintain intracellular ATP levels (shown by increased oxygen consumption). This permits us to study the changes in intracellular NADH during changing steady states of work output under rigorously controlled conditions of the extracellular environment. We tested the hypothesis that changing intracellular Ca^{2+} levels during changing work rates regulates the steady-state level of mitochondrial NADH via stimulation of mitochondrial dehydrogenases. Endogenous fluorescence emission from heart cells comes mainly from total mitochondrial reduced nicotinamides, with negligible contribution from cytosolic pyridine nucleotides (Nuutinen, 1984). The extent of reduction of mitochondrial NAD(P) is in near equilibrium with the extent of reduction of other components of the electron transport chain as well as with $\Delta\mu$ (Ugurbil and From, 1993). The extent of reduction of these pools is governed by nonequilibrium inputs and outputs to the electron transport chain. The nonequilibrium inputs and outputs are 1) replenishment of oxygen supply, 2) replenishment of substrate, and 3) dissipation of the electrochemical gradient across the inner mitochondrial membrane, which in turn is governed by the rate of ATP utilization. In the studies reported here, oxygen supply was not limiting (White and Wittenberg, 1993) and substrate supply included both glucose and fatty acid. This enabled us to study the consequences of interventions that change intracellular calcium levels on the extent of reduction of mitochondrial NAD(P)H during a standardized increase in ATP utilization. Our experiments provide a direct measure of changes in the steady-state NADH levels of heart cells before, during, and after pacing. A reversible significant decrease in the NADH level of ~30% of the initial level is observed when cells are paced at 5 Hz. Under experimental conditions that affect the initial NADH level (e.g., absence of β-OH butyrate substrate or presence of ruthenium red), the absolute magnitude of the decrease is not significantly different, but the direction of reversible significant change is always the same when the cells contract specifically in response to repetitive stimulation.

The isolated heart cell model

There are several advantages to the use of single, functionally intact isolated heart cells for the study of the effects of calcium on mitochondrial NADH during electrical pacing. The heart cells are architecturally and metabolically intact, with maintenance of intracellular compartmentation and energy reserves as in the whole heart. Oxygen supply can be maintained at nonlimiting rates as the pacing rate is increased; that is not possible in the whole perfused heart, where oxygen supply is modulated by vascular and humoral controls. Note that Zhang et al. (1995) showed that at high work loads, the beating in situ heart is limited in its energy production by the availability of blood flow and oxygen supply. Accordingly, we are able to study the interactions of changes in intracellular calcium and mitochondrial NADH, without complications due to insufficient oxygen supply. We assume that in the whole heart there may be modulation of the basic reactions described in its component cells, and throughout the discussion we relate our findings (where possible) to those described in the blood-perfused whole heart, which is a more integrated model. Although work

output in the unloaded heart cells is considerably less than that of the whole heart, oxygen uptake can be stimulated up to sevenfold by pacing. Our basic finding that increased intracellular calcium during pacing does not increase the level of mitochondrial NADH when oxygen supply is not limiting must be as valid for the whole heart as it is for isolated heart cells.

The effect of oxygen supply on mitochondrial NADH

Mitochondrial NADH reflects the oxygen supply to the mitochondria, and mitochondrial NADH levels increase as the oxygen supply to the heart cells is diminished (White and Wittenberg 1993). NADH builds up as a consequence of the decreased availability of oxygen to react with cytochrome oxidase, a rate-limiting step in electron transport (see Fig. 6). During electrical pacing, oxygen consumption of the heart cells is increased sevenfold (see Fig. 5) to maintain mitochondrial oxidative phosphorylation at a rate commensurate with the rate of ATP utilization. Note that a 20-fold increase in respiration is observed when the electrochemical gradient is dissipated with 8 μM carbonyl cyanide-*m*-chlorophenylhydrazone, a proton ionophore. Therefore the respiratory stimulation we observe is ~30% of maximum respiration. During 5 Hz stimulation, the measured oxygen levels are always above 20 mm Hg, as measured by the oxygen electrode. In the isolated cell system, with short diffusion distances, mitochondrial oxygen uptake becomes limited only below 5 mm Hg (Wittenberg and Wittenberg 1985). Under the present conditions of abundant oxygen supply, we observe a 30% decrease in NADH levels during (increased) pacing at 5 Hz. The decrease in NADH levels during increased pacing is in accord with experiments in whole heart by Conley et al. (1991), who took great care to limit their observations to aerobic regions of the working heart, and by Scott et al. (1994). Previous reports (Katz et al., 1987) that mitochondrial NAD(P)H of the beating heart is progressively increased with increased work output probably reflect compromised oxygen delivery to the area under observation (White and Wittenberg, 1993; Heineman and Balaban, 1993) rather than an effect of increased intracellular calcium on activation of mitochondrial dehydrogenases.

The effect of substrate supply on mitochondrial NADH

The heart preferentially uses fatty acid substrates, augmented by glucose, during work; ketone bodies (such as β-OH butyrate) and glucose are normally present in blood. We show here that the addition of β-OH butyrate as a substrate substantially increases mitochondrial NAD(P)H levels (Fig. 1) and PCr/ATP in resting and stimulated heart cells (see Table 1). The observed increase in high energy phosphates is in accord with data reported by Kim et al. (1991) using dog heart. Accordingly, β-OH butyrate augmented by glucose was used in all subsequent experiments reported here. Despite the increase in mitochondrial NAD(P)H observed at rest in the presence of β-OH butyrate, we saw a reversible decrease in mitochondrial NAD(P)H of ~0.10 of total mitochondrial NAD(P) during pacing at 5 Hz in these well oxygenated cells. Possibly the β-OH butyrate dehydrogenase reaction specifically influences the supply and regulation of reducing equivalents to NADH. At steady state, the rate of inflow of reducing equivalents to NADH must equal the rate of outflow, and in addition must maintain the level of the NADH pool. Because NADH levels are higher in the presence of glucose plus β-OH butyrate, the rate of supply of NADH is greater in this condition than in the presence of glucose alone.

The effect of the rate of ATP utilization on mitochondrial NADH

During pacing, sarcoplasmic calcium levels increase, initiating contraction and stimulating ATP utilization at the myofilaments. Change in the extent of reduction of mitochondrial NAD(P) reflects change in the magnitude of $\Delta\mu$ (Ugurbil and From, 1993). The $\Delta\mu$ is the sum of the mitochondrial membrane potential and the pH gradient across the mitochondrial membrane. In the presence of carbonyl cyanide-*m*-chlorophenylhydrazone, NADH becomes fully oxidized, and the measured value of NADH drops to zero. We showed previously that pacing at 5 Hz does not change cytoplasmic pH as measured with an intracellular pH indicator (White and Wittenberg, 1993), so the measured decrease in NADH levels must reflect a change in $\Delta\mu$.

Wan et al. (1993) reported that increased pacing of whole heart, sufficient to increase respiration fourfold, decreased the mitochondrial membrane potential measured with voltage-sensitive dyes. This is consistent with the decreased NAD(P)H that we observed in rapidly paced cells with a six- to sevenfold increase in respiration.

The ability of the cells to withstand the stimulation protocol without significant change in the PCr/ATP level shows that the energy status of the cells is maintained. These observations suggest that at increased work a new steady-state balance is achieved between the rate of substrate supply to the electron transport chain and the rate of utilization of $\Delta\mu$, whereas ATP and ADP levels are maintained.

The effect of changes in intracellular calcium on mitochondrial NADH

Increased mitochondrial calcium during pacing

During electrical pacing of isolated heart cells, sarcoplasmic and mitochondrial Ca^{2+} (Ca_i) are substantially increased to ~750 nM, and the active form of pyruvate dehydrogenase is increased to 50% of total (Di Lisa et al., 1993). The activity

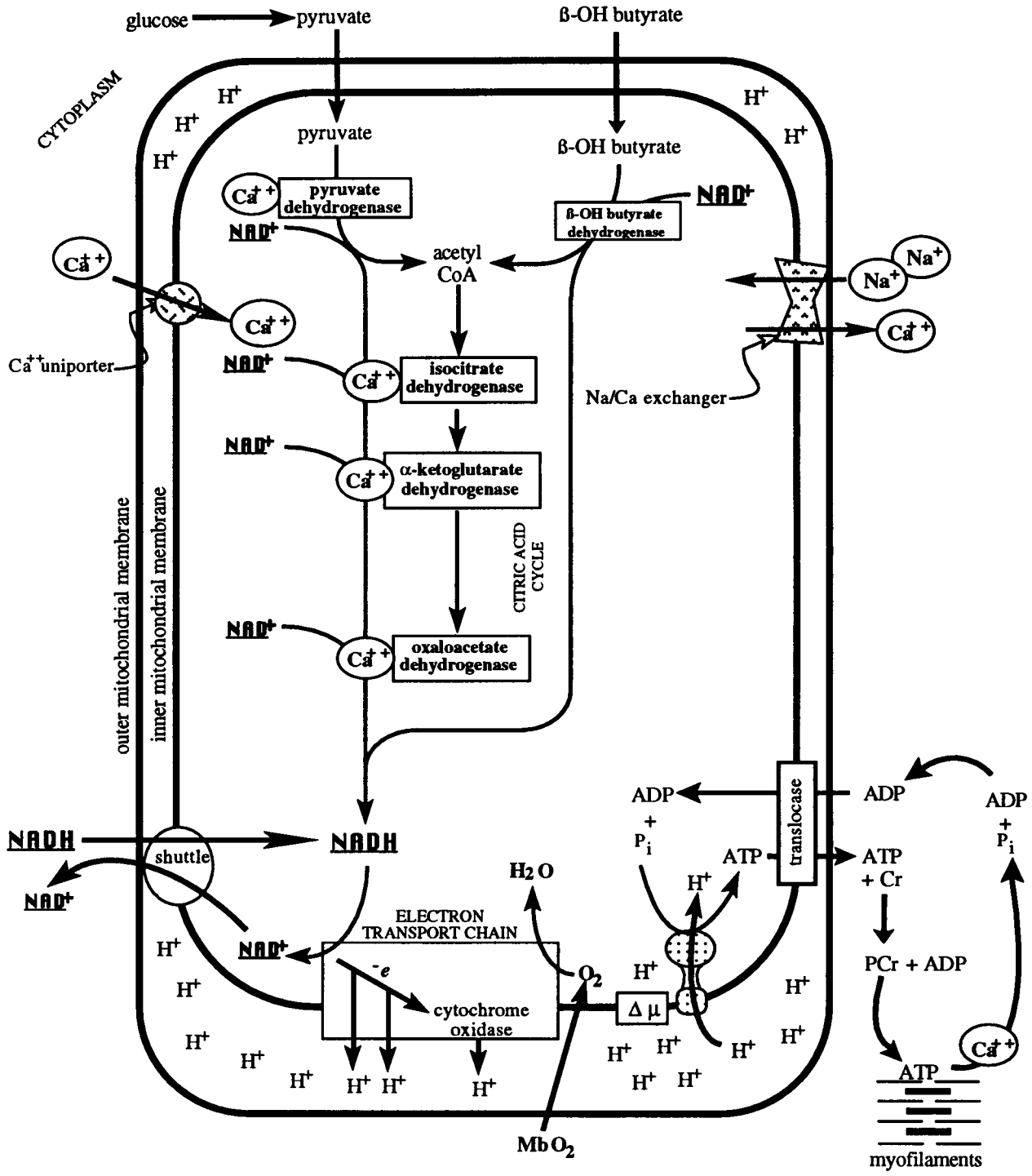

FIGURE 6 Some relevant mitochondrial reactions during pacing. Two of the reactions shown operate far from equilibrium in paced cells. These are the reaction of cytochrome oxidase with oxygen and the reaction of F_0F_1ATP synthase with $\Delta\mu$, ADP, and P_i (Ugurbil and From, 1993). Other reactions of the electron transport chain operate near equilibrium. In steady state, NADH concentration is linked to $\Delta\mu$, such that the decreasing NADH concentration reflects decreased $\Delta\mu$. The major net reaction of the electron transport chain is oxidation of NADH by oxygen delivered by myoglobin (MbO_2) at the outer mitochondrial membrane, with generation of $\Delta\mu$ across the inner mitochondrial membrane. $\Delta\mu$ is dissipated by ATP synthesis. Repetitive pacing at 5 Hz causes a sevenfold increase in respiratory oxygen uptake. The oxygen reacts with cytochrome oxidase at the inner mitochondrial membrane, electron transport along the mitochondrial chain is enhanced, and there is increased proton translocation across the mitochondrial membrane to augment $\Delta\mu$. The increased activity of the reactions of the electron transport chain and ATP synthase tend to decrease NADH by oxidation to NAD^+. In turn, NADH is replenished by dehydrogenase reactions. Intracellular calcium increases during pacing, increasing ATP utilization at the myofilaments, and mitochondrial calcium is increased by calcium entry through the Ca^{2+} uniporter. Four mitochondrial dehydrogenases (*bold type in boxes*) that generate NADH are activated by calcium so that the rate of delivery of NADH to the electron transport chain can be enhanced.

of several other mitochondrial dehydrogenases indicated in Fig. 6 are enhanced by increased calcium, and the rate of delivery of reducing equivalents to NADH can be increased to meet the increased demand for electron transport. It was suggested that Ca^{2+} activation of dehydrogenase activity might be expected to increase mitochondrial NAD(P)H levels (McCormack et al., 1990). Our results, under conditions in which oxygen supply is not limiting (Figs. 1 and 3), show that steady-state mitochondrial NAD(P)H decreases during rapid pacing of isolated myocyte.

Potassium channel blockers were shown to depolarize heart cells and to broaden the cardiac action potential (Giles and Imaizumi, 1988). Depolarization would reduce the potential energy available for Na/Ca exchange, and Ca_i levels would be expected to increase. Low Na^+ medium increases Ca_i (Doeller and Wittenberg, 1990; Gupta and Wittenberg, 1993), but we showed previously that there was no increase in mitochondrial NAD(P)H (White and Wittenberg, 1993). In that system, electrical pacing was not possible. In this report, we show that when Ca_i is expected to further increase by 5-Hz pacing in the presence of K^+ channel blockers, mitochondrial NAD(P)H decreases to a significantly greater extent than control (Fig. 3). Thus mitochondrial calcium levels increase during pacing, and the activity of mitochondrial dehydrogenases is said to be enhanced without leading to a buildup of mitochondrial NADH.

Inhibition of contraction: ryanodine and verapamil

Ryanodine (at 5 μM) maintains sarcoplasmic reticulum calcium channels in the open position, thus abolishing calcium-induced calcium release (Hille, 1984). Under these conditions, the cells do not contract in response to electrical stimulation. In contrast to control cells, mitochondrial NAD(P)H does not change during rapid stimulation (Fig. 2).

Verapamil plus $NiCl_2$ (which blocks L-type and T-Type Ca^{2+} channels, respectively) inhibits entry of extracellular Ca^{2+} into the sarcoplasm (Hille, 1984). We find that verapamil plus $NiCl_2$ prevents contraction and the marked drop in mitochondrial NAD(P)H normally observed during 5 Hz pacing in single myocytes. Our findings are in accord with the report of Scott et al. (1994) that blocking increased work output in the perfused heart with 2,3-butanedione monoxime also blocked the decrease in mitochondrial NAD(P)H. We conclude that electrical stimulation that does not lead to contraction of heart cells does not change their mitochondrial NAD(P)H levels.

Decreased mitochondrial calcium: ruthenium red

In this study we use ruthenium red to specifically block calcium entry into mitochondria via the Ca^{2+} uniporter (see Fig. 6; Moore, 1971; Gunter and Pfeiffer, 1990). Ruthenium-red treatment has multiple consequences on cardiac mitochondrial function: 1) cytoplasmic calcium levels are maintained, (Hansford 1994); 2) calcium-dependent activation of mitochondrial dehydrogenases is blocked (McCormack and England, 1983; Hansford 1987); 3) activation of mitochondrial ATPase/synthase during pacing is blocked (Harris and Das, 1991); and 4) PCr levels decrease and ADP levels increase in response to increased work load (e.g., Katz et al., 1988; Unitt et al. 1989). In our heart cell model, however, the PCr level does not decrease significantly during stimulation sufficient to stimulate oxygen consumption sevenfold (See Table 1). This indicates a stable energetic status, which may be attributed to the high nonlimiting oxygenation levels that we are able to achieve in the heart cell perfusion. Thus the rate of oxidative phosphorylation is sufficient to maintain PCr levels at increased energy demand. Zhang et al. (1995) recently reported that in the blood-perfused beating heart at high work loads, the ATP synthesis rate was limited by blood flow and oxygen supply.

At the dose used here, we found that ruthenium red-treated cells contract synchronously in response to electrical stimulation, indicating that excitation-contraction coupling remains intact. Therefore sarcolemmal calcium channels and sarcoplasmic calcium-release channels are not blocked. Because Ca^{2+} entry into mitochondria is blocked at this dose of ruthenium red, we conclude that Ca^{2+} entry into mitochondria is not a prerequisite for contraction maintenance.

The NADH level of cells in the presence of ruthenium red is significantly lower than that of control cells paced at 0.5 Hz. Rapid pacing (5 Hz) brings about a further 25% decrease in mitochondrial NAD(P)H (see dark bars in Fig. 4) and a significant increase in oxygen consumption (see Fig. 5). Assuming that ruthenium red does not increase the rate of ATP utilization (as indicated by the oxygen consumption data), a plausible explanation might be that the rate of supply of reducing equivalents is decreased because of decreased activation of dehydrogenases, and a new lower steady-state level of NAD reduction is observed.

Mitochondrial calcium in cells paced at 0.5 Hz in the presence of β agonists is ~200 nM (Di Lisa et al., 1993). Our data strongly suggest that mitochondrial calcium at concentrations below 200 nM dominates the regulation of NAD(P) reduction.

SUMMARY

We tested the hypothesis that increased intracellular Ca^{2+} levels during increased work regulates the steady-state level of mitochondrial NADH by stimulation of mitochondrial dehydrogenases. Steady-state mitochondrial NAD(P)H of well oxygenated control cells (see Fig. 1 and the lightly shaded bars in Fig. 2) drops significantly and reversibly by ~30% during 5 Hz pacing. This response is blocked when contraction is blocked. The mitochondrial Ca^{2+} is known to increase from 200 nM to 700 nM during rapid pacing of heart cells. Mitochondrial Ca^{2+} must be below control levels in the presence of ruthenium red. Blocking the entry of Ca^{2+} into the mi-

tochondria does lead to a decrease in NADH levels compared with controls at all rates of pacing. Among other possibilities, this may imply that dehydrogenase activity dominates regulation of NAD(P)H levels in this range of mitochondrial calcium concentration.

The simplest explanation of these findings is that in the absence of pharmacological intervention, mitochondrial ATP synthase activity is stimulated by increased ATP demand at the myofilaments during pacing. The rate of substrate supply to the electron transport chain during pacing must be increased sufficiently to maintain ATP levels but at a new lower steady-state level of NADH. In this situation, other factors may override dehydrogenase control of the NAD(P)H level.

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