Biochemical Pharmacology, Vol. 56, pp. 173–179, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00016-1

NADH Fluorescence in Isolated Guinea-Pig and Rat Cardiomyocytes Exposed to Low or High Stimulation Rates and Effect of Metabolic Inhibition with Cyanide

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ABSTRACT. In this study we investigated whether NADH fluorescence levels changed in response to low or high rates of electrical stimulation in single ventricular myocytes isolated from rat and guinea-pig hearts, either during a single contraction or upon sustained electrical stimulation of cells. NADH levels were determined from cell autofluorescence and cell length monitored using an edge-tracking device. NADH/NAD^+ was obtained by addition of cyanide, 100% NADH, and carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone (FCCP), 100% NAD^+. Rat myocytes exhibited slightly higher resting fluorescence levels than guinea-pig cells; however, NADH/NAD^+ was higher in rat than guinea-pig cells ($P < 0.05$), 24.3 ± 4.3 (N = 17) vs 14.6 ± 1.6 (N = 17), respectively. There was no change in NADH fluorescence during a single contraction when cells were stimulated at either low (0.2 Hz) or high (3 Hz) rates in either species. Furthermore, NADH levels did not change upon sustained stimulation at 3 Hz in either species. Metabolic blockade with cyanide induced a dose dependent rise in NADH fluorescence which was similar for both rat and guinea-pig myocytes and reached a maximum at ≥ 1 mM of cyanide. Although a full recovery of NADH fluorescence was seen in both types of cells after brief exposure to cyanide, the rate of recovery was significantly slower in rat myocytes; times to 90% recovery were 110 ± 29 sec, N = 6, and 264 ± 50 sec, N = 6, for guinea-pig and rat cells, respectively. This work demonstrates that although rat and guinea-pig myocytes have different resting NADH/NAD^+, their response to electrical stimulation is the same, whereas in response to metabolic inhibition subtle differences are seen. BIOCHEM PHARMACOL **56**;2:173–179, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cardiomyocytes; cyanide; NADH; mitochondria; metabolic inhibition; autofluorescence

Mitochondrial function in heart cells is essential for the normal cycle of excitation, contraction and relaxation, and also in determining the extent of recovery following various cardiac insults [1–5]. Alterations in mitochondrial function may be one reason for the observed species differences in recovery from insults like hypoxia [6, 7] or the calcium paradox [8, 9]. Normal cardiac function like, excitation-contraction coupling and contribution of sarcolemmal Ca^{2+} transport pathways, also varies between species [10] and mitochondrial Ca^{2+} transport may also differ [11–13]. The latter is important since mitochondrial Ca^{2+} is now thought to play an integral role in regulation of the intramitochondrial dehydrogenases which are responsible for NADH and hence ATP production in mammalian mitochondria (NADH is the primary electron source for electron transport chain in oxidative phosphorylation) [14]. In several types of mammalian cells and isolated mitochondria, changes in cell autofluorescence (at wavelengths between 420–540 nm when excited at 350 nm) have been positively correlated with changes in mitochondrial NADH levels [15–18]. Although this signal does not distinguish between NADH and NADPH or between cytosolic and mitochondrial NADH, at least 80% of the autofluorescence has been shown to originate from mitochondrial NADH in the heart [16].

NADH levels have been shown not to change in single rat myocytes in response to a single contraction [18, 19], unlike in isolated trabeculae where a decrease was seen [20]. Upon sustained electrical stimulation, NADH levels have been reported to decrease [18] or increase [19] in isolated rat cells. No studies have been performed on isolated guinea-pig myocytes. The aims of this study were to determine whether NADH fluorescence changes could be detected in myocytes from either species under physiological conditions (37° and 2 mM of Ca^{2+}) at rest, during a single contraction or upon sustained electrical stimulation and whether cells from both species responded similarly to metabolic blockade. Part of this work has appeared as an abstract [21].

MATERIALS AND METHODS

Myocyte Isolation

Rat and guinea-pig ventricular myocytes were isolated by collagenase digestion based on the method of Hancox and Levi [22]. However, in order to improve the yield of

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† *Abbreviation:* FCCP, carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone.

Received 26 June 1997; accepted 12 January 1998.

rod-shaped cells for both species, the method was modified. The basic perfusion medium (solution A) contained (mM): NaCl 137; KCl 5; $MgSO_4.7H_2O$ 1.2; HEPES 20; Glucose 16; Na pyruvate 5; $MgCl_2$ 1.8; adjusted to pH 7.25 with NaOH. Animals (weights 220–270 g) were killed by cervical dislocation, the hearts removed and immediately perfused with solution A plus 0.75 mM of $CaCl_2$ at a flow rate of 10 mL/min for 4 min. The heart was then perfused with solution A containing 90 mM of EGTA for 4 min before switching over to the enzyme solution which contained solution A plus 15 μM of $CaCl_2$, 1.0 mg/mL collagenase (Worthington Type I, Worthington Biochemical Corporation) and 0.1 mg/mL protease (Sigma type XIV). After 1 min perfusion at 10 mL/min the flow rate was reduced to 7 mL/min. The enzyme solution was recirculated for 5 min in the guinea-pig whereas for the rat it was recirculated for approximately 15 min (until the tissue became soft). The enzyme was then washed out with solution A containing 150 mM of $CaCl_2$ for 4 min at a flow rate of 10 mL/min. The ventricles were then removed, sliced approximately 10 times and shaken at 37° for 5 min in solution A + 150 mM of $CaCl_2$. Cells were filtered and allowed to sediment at room temperature for 7 min. The supernatant was aspirated and cells resuspended in solution A containing 0.5 mM of $CaCl_2$. The sedimentation was repeated and the myocytes finally resuspended and stored at room temperature in solution A with added 1 mM of $CaCl_2$. Isolated myocytes were used within 8 hr.

Experimental Procedure

Myocytes were placed in a Perspex chamber and continuously superfused at a constant flow of 3.5 mL/min. Myocytes were perfused with normal Tyrode solution containing (mM): NaCl 137; KCl 5; $MgSO_4 \cdot 7H_2O$ 1.2; NaH_2PO_4 1.2; glucose 16; HEPES 20; $CaCl_2$ 2.0; pH 7.4. Myocytes were viewed with the aid of a TV camera attached to Nikon Diaphot inverted microscope (Nikon) and illuminated with the microscope light through a low-pass filter. Cells were field stimulated using a Grass (SD 9) stimulator and silver/silver chloride electrodes.

Measurement of Cell Fluorescence and Cell Length

The epifluorescence of single myocytes was measured with a photon counting system (Newcastle Photometric Systems) attached to a Nikon Diaphot inverted microscope as described previously [5]. NADH autofluorescence was excited at 350 nm and the emission was measured between 400–520 nm. However, intracellular NADH cannot be calibrated in absolute terms and so was expressed as either % resting levels in quiescent myocytes or $NADH/NAD^+$ (see Results for calculation) [5, 19].

Cell length was monitored simultaneously with cell fluorescence using an edge-tracking device.

Materials

Sodium cyanide and other basic chemicals were obtained from Sigma.

Analysis of Results

Results are expressed throughout as means ± SEM unless indicated otherwise. Statistical significance was analysed using Student's *t*-test (paired where appropriate) or ANOVA.

RESULTS

Effect of Changes in Stimulation Rate on NADH Fluorescence

NADH fluorescence and cell length were monitored simultaneously in either rat or guinea-pig myocytes as described in Methods. Figure 1 show a typical response of a single guinea-pig myocytes to stimulation at either 0.2 or 3 Hz. It is apparent that NADH fluorescence does not change during a single contraction at either the low or high rate. Identical results were observed for rat myocytes.

Effect of Exposure to and Washout of CN on NADH Fluorescence

In Fig. 1, the raw data for NADH fluorescence (in arbitrary units) is presented. To estimate the NADH to NAD^+ ratio ($NADH/NAD^+$) of cells, exposure to CN^- and FCCP† has been used [16–19]. To determine the concentration of CN^- required to give maximum increase in NADH levels (complete inhibition of cytochrome c oxidase) under the present conditions, cells were exposed to different concentrations of CN^-. Figure 2 shows the dose response curve for the effect of CN^- on NADH autofluorescence for both rat and guinea-pig heart cells. It is evident that for both types of cells, a maximum response was obtained at a CN^- concentration of $\geq$1 mM. Furthermore the maximum increase in NADH was approximately 180% for guinea-pig and slightly but not significantly lower for rat cells. The maximum response to CN^- is consistent with maximum inhibition of the electron transport chain, an effect we have confirmed previously using other electron transport chain inhibitors [5] and by the effect of rotenone, an irreversible inhibitor of NADH dehydrogenases: rotenone (2 μg/mL) gave the same maximal effect as CN^- (the percentage increase was 179 ± 16 for rat and 180 ± 14 for guinea-pig, N = 6–7).

The effect of a short exposure to CN^- on NADH has been reported to be fully reversible in myocytes, however, in our experiments we found that although the effect was reversible for a 1 min exposure, there were significant differences in the response between rat and guinea-pig cells: Fig. 3 shows a typical response to CN^-. The time to peak response (Tpk) and actual peak levels were very similar for the two species (Fig. 4); Tpk was 50 ± 5 sec for rat vs 67 ±

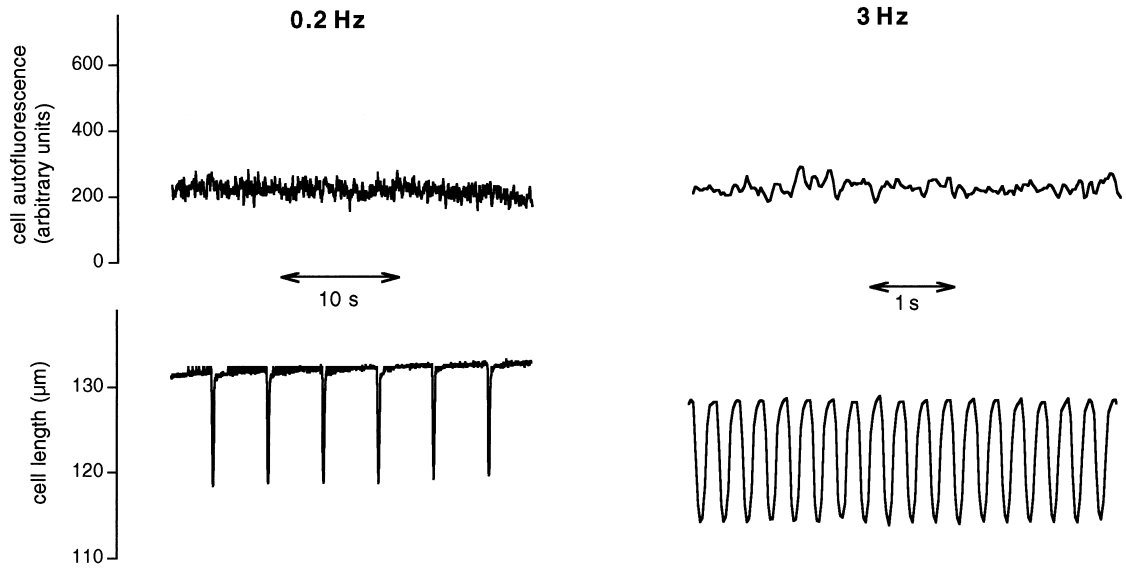

FIG. 1. Changes in NADH fluorescence and cell length in response to electrical stimulation. Data from a single guinea-pig myocyte is shown when the cell was stimulated to contract at either 0.2 Hz (left panel) or 3 Hz (right panel; note the expanded timescale).

10 sec for guinea-pig and maximum response was 163 ± 10% for rat vs 186 ± 6% for guinea-pig. The Tpk response to CN^- in both rat and guinea-pig cells was the same when measured at 37° or 28°. However upon washout there was a very clear difference; the NADH levels in guinea-pig cells returned much more rapidly than in rat cells (Figs. 3 and 4). In guinea-pig cells the time to reach 50% and 90% recovery was 61 ± 9 and 110 ± 29 sec respectively (37°; N = 6) whilst in rat cells the recovery was much slower (169 ± 34 and 264 ± 50 sec respectively, 37°; N = 6). This difference was further exaggerated at the lower temperature of 28° (Fig. 4B). To determine whether this effect was peculiar to CN^-, we investigated the effect of another cytochrome c oxidase inhibitor, sodium azide. At 20 mM, sodium azide gave the same maximum response as CN^- and again the recovery was slower in rat cells suggesting that the effect is specific to inhibition of cytochrome c oxidase (data not shown).

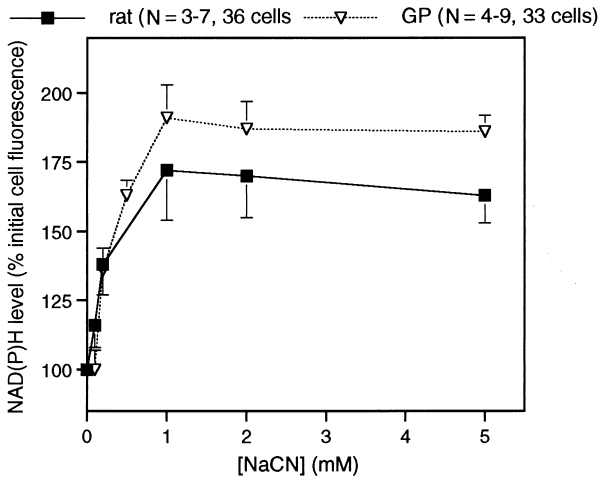

FIG. 2. Dose response for the effect of CN^- on NADH in quiescent cardiomyocytes. Dose dependent response to CN^- as measured by changes in NADH autofluorescence in single rat (closed squares) and guinea-pig (open triangles) heart cells superfused at a constant flow of 3.5 mL/min at 37°. Quiescent cell autofluorescence was taken as 100%. Maximum response to CN^- was measured and expressed as % of initial fluorescence for each cell. Values are mean ± SE (33–36 cells from 3–9 hearts).

Effect of Sustained Electrical Stimulation on NADH/NAD$^+$

NADH/NAD^+ was determined by exposing cells to CN^- and then FCCP. A typical response is shown in Fig. 5. Note that the cell enters a rigor state (indicated by cell shortening) upon continued exposure to FCCP only after fluores-

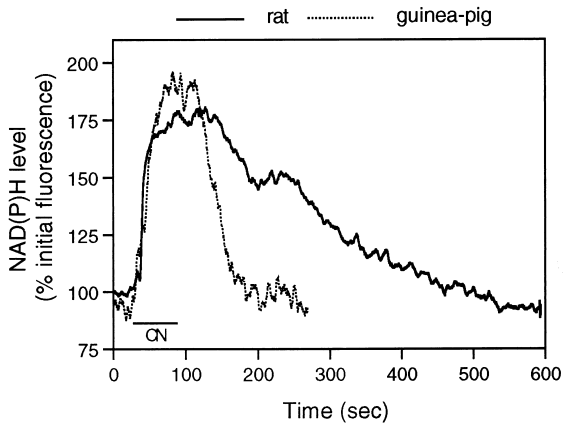

FIG. 3. Effect of short exposure to CN^- on NADH levels. The figure shows a representative trace showing the effect of short exposure of 5 mM of CN^- on NADH autofluorescence in quiescent single cells isolated from rat or guinea-pig hearts. Resting NADH autofluorescence was taken as 100%. Mean values are shown in Fig. 4.

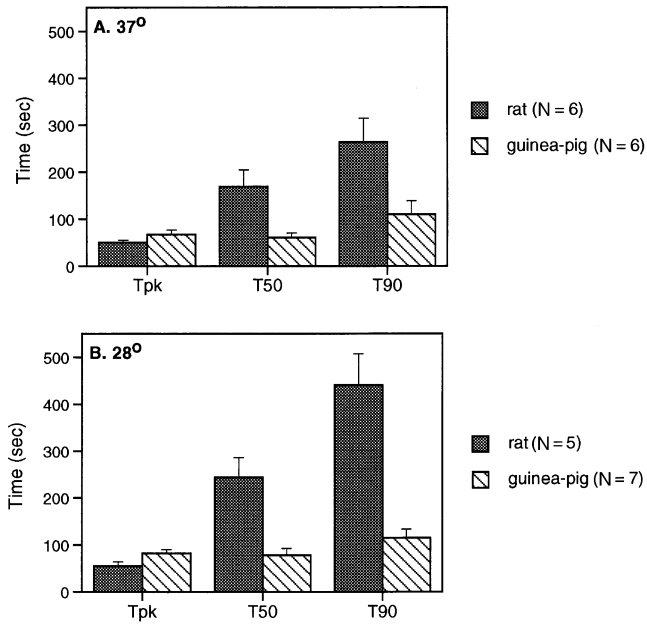

FIG. 4. Effect of short exposure to CN^- on NADH levels; mean values. Mean values for times to peak response (Tpk) and times to 50 and 90% recovery (T50 and T90, respectively) of the fluorescence signal following 1 min exposure to 5 mM of CN^- and measured at either 37° (B) or 28° (C). There was no difference in TpK between rat and guinea-pig responses. Recovery times at both T50 and T90 were significantly higher for the rat when measured at 37° ($P < 0.05$) or at 28° ($P < 0.005$).

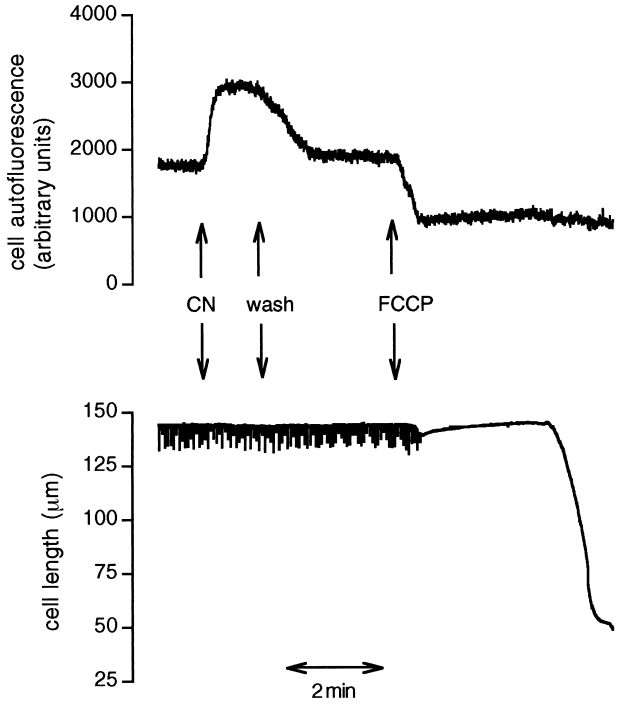

FIG. 5. Effect of exposure to CN^- and FCCP on NADH fluorescence and cell length. The trace shows simultaneous measurements of NADH fluorescence and cell length changes in a single rat myocyte exposed to 5 mM of CN^- and 1 μM of FCCP.

cence has reached a minimum; this is due to ATP depletion by uncoupling of the respiratory chain by FCCP. Resting autofluorescence levels were slightly but not significantly higher in rat myocytes (the autofluorescence of single myocytes was roughly equivalent to that observed with a solution of free NADH of 5 μM) despite cell length being similar between the two species (Fig. 6). However, resting NADH/NAD^+ was approximately 70% higher in rat cells than guinea-pig cells ($P < 0.05$), 24.3 ± 4.3 (N = 17) vs 14.6 ± 1.6 (N = 17), respectively (Fig. 6).

Figure 1 showed that NADH fluorescence did not change during a single contraction at either 0.2 Hz or 3 Hz in cells from either species. To determine whether NADH changed upon prolonged stimulation at 3 Hz, cells were stimulated at this rate for 2 min before return to low stimulation rate for 3 min. Figure 7 shows the fluorescence and cell length changes in a single cell in response to this protocol, with mean data expressed as NADH/NAD^+ shown in Fig. 8. NADH levels remained constant in rat myocytes throughout this protocol. The NADH/NAD^+ is slightly but not significantly lower in guinea-pig cells than in rat cells and there is no significant difference in the overall response (using ANOVA). Again there is no change in NADH during sustained stimulation, however, in guinea-pig cells there is a significant fall in NADH/NAD^+ 3 min after return to low stimulation rate, 18.0 ± 2.3 to 13.3 ± 3.4, $P < 0.05$.

DISCUSSION

The results presented here demonstrate that NADH levels of rat and guinea-pig myocytes did not change during a single contraction at either low or high rates of electrical stimulation. Resting NADH/NAD^+ was higher in rat then guinea-pig cells, which may reflect differences in mitochondrial metabolism, but in neither species was there a change in NADH/NAD^+ upon prolonged exposure to high stimulation. In rat myocytes there was also no change upon return to low stimulation whereas in guinea-pig cells a significant decrease in NADH/NAD^+ was observed. Furthermore, in calculating NADH/NAD^+ we noted that the effect of CN^- on NADH fluorescence differed between the two species: although the concentration of CN^- required for complete respiratory chain inhibition and rate of increase in NADH fluorescence was similar for both cell types, the rate of recovery of fluorescence was much slower in rat cells. Because sodium azide gave the same response as CN^-, this suggests that the effect is specific to inhibition of cytochrome c oxidase, possibly reflecting a slower dissociation of CN^- from the enzyme in guinea-pig cells.

The possibility that the slower recovery of fluorescence in guinea-pig cells was due to changes in either intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or intracellular pH (pH_i) was also considered because $[Ca^{2+}]_i$ is known to activate dehydrogenases of the citric acid cycle and to increase NADH levels [14] and inhibition of aerobic metabolism with CN^- will increase lactate output via glycolysis. Although lactate

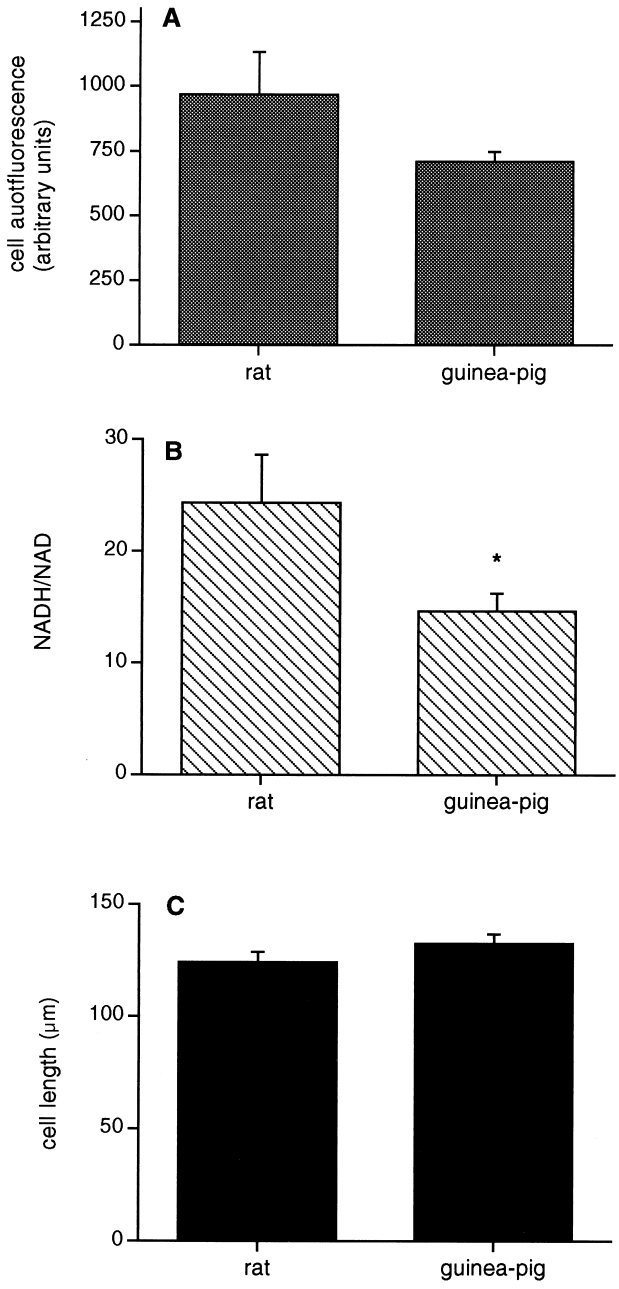

FIG. 6. Basal values for NADH fluorescence, NADH/NAD$^+$, and cell length. Panel A: cell autofluorescence levels (arbitrary units); Panel B: NADH/NAD$^+$ (obtained using CN$^-$ and FCCP, see text); Panel C: cell lengths. The parameters were measured in the same cells; N = 17 cells from 4 hearts for both species. *$P < 0.05$ for NADH/NAD$^+$; other differences are not significant.

may not accumulate as the cells are being continuously superfused, the rate of transport of lactate out of the cells is known to differ between rat and guinea-pig myocytes [23]. This difference might alter intracellular pH to different extent which, in turn, would affect the equilibrium between NADH and NAD$^+$. However, there was no change in either $[Ca^{2+}]_i$ or pH_i following 1-min exposure to 5 mM of CN$^-$ in either rat or guinea pig cells (data not shown).

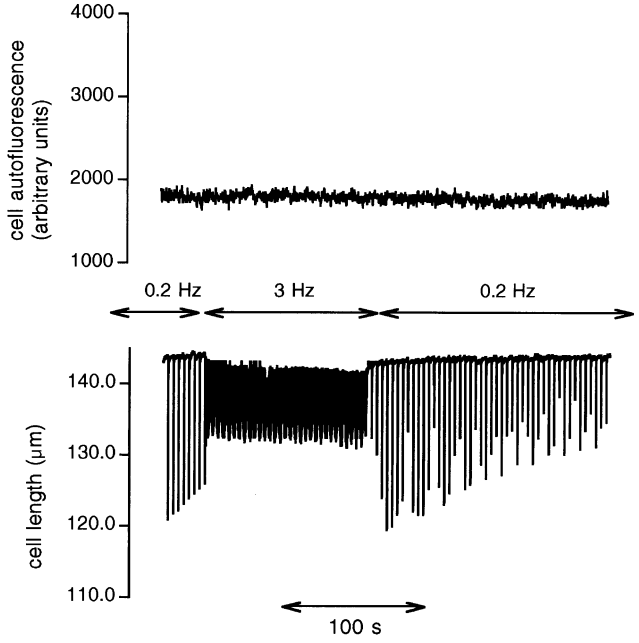

FIG. 7. NADH fluorescence and cell length changes in response to sustained stimulation at 3 Hz. Data from a single rat myocytes is shown.

A comparison of changes in NADH autofluorescence in myocytes isolated from rat and guinea-pig hearts is important because both species are widely used to study mitochondrial function in intact cells [5, 16, 18, 19, 24]. This is normally carried out in different laboratories under different conditions using different protocols, thus making comparisons between the two more difficult. Mitochondrial NADH provides fuel for the electron transport chain to generate the chemiosmotic gradient that drives the synthesis of ATP. As a result the NADH/NAD$^+$ has been widely used as a measure of the energy flux of a cell [see 5]. Resting NADH/NAD$^+$ was higher in rat then guinea-pig cells,

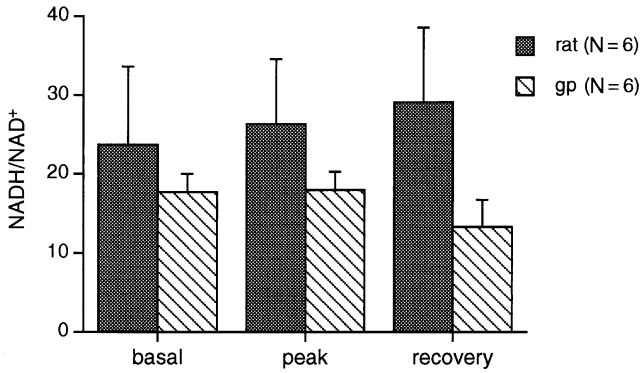

FIG. 8. NADH/NAD$^+$ upon sustained electrical stimulation of myocytes. The figure shows mean values of the experiments shown in Fig. 7 for initial stimulation at 0.2 Hz (basal), 2 min stimulation at 3 Hz (peak) and 3 min after return to 0.2 Hz (recovery). There are no significant differences between species; rat cells show no change throughout the whole protocol but for guinea-pig cells there is a significant decrease upon return to low stimulation, peak vs recovery, $P < 0.05$.

which may reflect differences in mitochondrial content and/or metabolism and may also account for the slower recovery of fluorescence in rat cells upon washout of CN^-. It is interesting to note that basal NADH levels tended to be higher in rat cells, though not significantly, which could be due to the fact that the mitochondrial content of cardiomyocytes is related to heart rate, being higher in species with faster heart rates. It is also possible that a relatively increased levels of citric acid cycle flux in the rat may be responsible for the higher NADH/NAD^+ enabling it to cope with energy demands of a beating heart.

Consistent with previous reports using rat myocytes [18, 19], NADH levels do not change during a single contraction at either low or high rates of electrical stimulation (Fig. 1). This was also the case for guinea-pig cells, suggesting that differing contributions of both sarcolemmal and mitochondrial Ca^{2+} transport pathways during the cell contractile cycle between the two species [10–12] do not necessarily influence the NADH response to stimulation. We also found that NADH/NAD^+ did not change in response to sustained electrical stimulation in cells from either species (Figs. 7 and 8). This is in contrast to previous work showing either an increase [19] or decrease [18] in NADH fluorescence in response to rapid stimulation. Possible reasons for the different results are the more physiological conditions of our study (37° and 2 mM of external [Ca^{2+}]) compared to the other studies done at 24^0 and 1 mM of external [Ca^{2+}]; it is known that in guinea-pig cells NADH fluorescence is sensitive to changes in temperature [24]. Furthermore, increases in the amplitude of both the Ca^{2+} transient and contraction of cells occurs at lower temperatures [25]. This in turn would increase mitochondrial [Ca^{2+}] which may alter the steady-state level of NADH by differential activation by Ca^{2+} of NADH producing and NADH utilising pathways, for example activation of dehydrogenases or ATP synthase, respectively [14, 26–28]. Further work is required to establish the exact relationship between changes in temperature, stimulation rate, intramitochondrial [Ca^{2+}] and NADH levels.

This work was supported by grants from the National Heart Research Fund and the Garfield Weston Trust. E. J. Griffiths is a recipient of a British Heart Foundation Intermediate Fellowship. We would like to thank Mrs. Dawn Wallace for excellent technical assistance.

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