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NADH supplementation decreases pinacidil-primed $I_{K(ATP)}$ in ventricular cardiomyocytes by increasing intracellular ATP

[1]Brigitte Pelzmann, [2]Seth Hallström, [1]Peter Schaffer, [1]Petra Lang, [2]Karl Nadlinger, [2]George D. Birkmayer, [3]Karoline Vrecko, [3]Gilbert Reibnegger & *,[1]Bernd Koidl

[1]Institut für Medizinische Physik und Biophysik, Karl-Franzens-Universität Graz, Harrachgasse 21, A-8010 Graz, Austria; [2]Department of Research and Development, Birkmayer Laboratories, Schwarzspanierstraße 15, A-1090 Wien, Austria and [3]Institut für Medizinische Chemie und Pregl Laboratorium, Karl-Franzens-Universität Graz, Harrachgasse 21, A-8010 Graz, Austria

1 The aim of this study was to investigate the effect of nicotinamide-adenine dinucleotide (NADH) supplementation on the metabolic condition of isolated guinea-pig ventricular cardiomyocytes. The pinacidil-primed ATP-dependent potassium current $I_{K(ATP)}$ was used as an indicator of subsarcolemmal ATP concentration and intracellular adenine nucleotide contents were measured.

2 Membrane currents were studied using the patch-clamp technique in the whole-cell recording mode at 36–37°C. Adenine nucleotides were determined by HPLC.

3 Under physiological conditions (4.3 mM ATP in the pipette solution, ATP_i) $I_{K(ATP)}$ did not contribute to basal electrical activity.

4 The ATP-dependent potassium ($K_{(ATP)}$) channel opener pinacidil activated $I_{K(ATP)}$ dependent on $[ATP]_i$ showing a significantly more pronounced activation at lower (1 mM) $[ATP]_i$.

5 Supplementation of cardiomyocytes with 300 $\mu g\,ml^{-1}$ NADH (4–6 h) resulted in a significantly reduced $I_{K(ATP)}$ activation by pinacidil compared to control cells. The current density was 13.8 ± 3.78 ($n=6$) *versus* 28.9 ± 3.38 pA pF^{-1} ($n=19$; $P<0.05$).

6 Equimolar amounts of the related compounds nicotinamide and NAD^+ did not achieve a similar effect like NADH.

7 Measurement of adenine nucleotides by HPLC revealed a significant increase in intracellular ATP (NADH supplementation: 45.6 ± 1.88 nmol mg^{-1} protein *versus* control: 35.4 ± 2.57 nmol mg^{-1} protein, $P<0.000005$).

8 These data show that supplementation of guinea-pig ventricular cardiomyocytes with NADH results in a decreased activation of $I_{K(ATP)}$ by pinacidil compared to control myocytes, indicating a higher subsarcolemmal ATP concentration.

9 Analysis of intracellular adenine nucleotides by HPLC confirmed the significant increase in ATP.
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Abbreviations: ($I_{K(ATP)}$), ATP-dependent potassium current

Introduction

The reduced nicotinamide-adenine dinucleotide (NADH) plays a central role for the energetic state of a cell. NADH carries electrons derived from catabolic reactions to their entry into the respiratory chain leading to the synthesis of ATP. This electron transfer results in the formation of NAD^+. The sum of NADH and NAD^+ is thought to be rather constant in a cell; thus the NADH/NAD^+ ratio is a crucial factor for its energetic state. Therefore, it is conceivable that oral application of NADH might positively affect the cellular energetic condition of humans.

Moreover, NADH serves as a cofactor for various enzyme reactions, further emphasizing the crucial role of NADH for numerous cell functions. Recent clinical studies have already demonstrated a positive effect of NADH treatment on patients suffering from Morbus Parkinson, chronic fatigue syndrome and depression (Birkmayer & Birkmayer, 1991; Birkmayer *et al.*, 1993; Kuhn *et al.*, 1996; Forsyth *et al.*, 1999). In addition, changes in NADH levels seem to correspond with stimulation and inhibition of neuronal metabolism, respectively (Rex *et al.*, 1999). NADH has also been identified as sensor of blood flow requirement in brain, muscle and other tissues (Ido *et al.*, 2001).

To test whether extracellular application of NADH affects the energetic state of a cell, we studied NADH-incubated cardiac ventricular myocytes by electrophysiological techniques.

The electrophysiological properties of a cardiomyocyte are strongly affected by its energetic condition. Especially, the ATP-dependent potassium current ($I_{K(ATP)}$) is known to link bioenergetic metabolism with membrane excitability by sensing intracellular concentrations of ATP and ADP. Under

*Author for correspondence; E-mail: bernd.koidl@uni-graz.at

physiological conditions, $K_{(ATP)}$ channels are predominantly closed because of inhibition by intracellular ATP (ATP_i) (Noma, 1983; Heidbüchel *et al.*, 1990; Schaffer *et al.*, 1999). However, when the ATP_i concentration falls below certain values (like in pathological states such as acute myocardial ischemia), the $K_{(ATP)}$ channels open. Certain drugs known as potassium channel openers (PCOs) are able to shift the ATP sensitivity of $K_{(ATP)}$ channels resulting in channel opening even at physiological levels of ATP_i (Arena & Kass, 1989; Nakayama *et al.*, 1990; Tseng & Hoffman, 1990; Pelzmann *et al.*, 2001). Thus, the amount of $I_{K(ATP)}$ activation induced by these drugs serves as an indicator of the cellular ATP content (Sasaki *et al.*, 2001).

Studying the PCO-primed $I_{K(ATP)}$, we report that supplementation of cardiomyocytes with NADH but not with the related compounds nicotinamide and NAD^+ results in a decrease of $I_{K(ATP)}$ activation by pinacidil. The NADH-induced decrease of $I_{K(ATP)}$ activation fits to the increase of intracellular ATP content measured by HPLC.

Methods

Myocyte isolation

Guinea-pig ventricular myocytes were isolated by Langendorff perfusion using collagenase as described previously (Piper *et al.*, 1982). The isolated myocytes were stored in Medium 199 (M2154, Sigma, St Louis, MO, U.S.A.), supplemented with 5 $\mu g\,ml^{-1}$ penicillin, 5 $IU\,ml^{-1}$ streptomycin, 0.5% glutamine and 5% fetal calf serum and were kept in an incubator at 37°C. Experiments were performed within 24 h after isolation.

Incubation procedure

The isolated myocytes were incubated with NADH (β-nicotinamide adenine dinucleotide, reduced form, disodium salt) and the related compounds nicotinamide and NAD^+ (β-nicotinamide adenine dinucleotide, oxidized form) in equimolar amounts 4 – 6 h before electrophysiological parameters and ATP content were evaluated.

Electrophysiological recordings and data analysis

Membrane currents were recorded using the whole-cell single-electrode voltage-clamp configuration of the patch-clamp technique (Hamill *et al.*, 1981) using a List L/M-EPC 7 amplifier (List, Darmstadt, Germany) as previously described (Pelzmann *et al.*, 2001). Myocytes were placed in an experimental chamber mounted on the stage of an inverted microscope (Axiovert, Zeiss, Oberkochen, Germany) and were superfused with standard extracellular solution (composition in mM: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 2.2, NaH_2PO_4 0.4, HEPES/Na^+ 10, D(+)-glucose 5.6, adjusted to a pH of 7.4 with NaOH) at 36 – 37°C with a flow rate of about 1.5 $ml\,min^{-1}$. When filled with standard internal solution (composition in mM: KCl 110, ATP/K^+ 4.3, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, HEPES/K^+ 10, adjusted to a pH of 7.4 with KOH) and placed into standard external solution, patch-pipette tip resistances were 1 – 3 MΩ. Only quiescent rod-shaped cells with clear cross striation were used for voltage-clamp experiments. Cell membrane capacitance (C_m) was determined by integration of the capacitive transient elicited by a 10 mV hyperpolarizing pulse from −50 mV. C_m (up to 100 pF) and series resistance (R_s, by at least 50%) were compensated. Voltage-clamp pulses were generated with a personal computer connected to a D/A and A/D converter (Digidata 1200, Axon Instruments, Foster City, U.S.A.). Data acquisition and analyses were performed using pCLAMP 5.7.1 software (Axon Instruments). In order to allow equilibration of the pipette solution with the cytosol, current recordings were started 5 min after rupture of the membrane patch.

Two experimental protocols were used. Modulation of $I_{K(ATP)}$ by drugs was evaluated as the change of outward current density (current amplitudes divided by C_m ($pA\,pF^{-1}$) in order to compensate for variations in cell size) at +30 mV in response to a 2 s ramp from −100 to +60 mV (I_{ramp}). Time course of $I_{K(ATP)}$ activation and blockade was studied by recording the holding current at −40 mV ($I_{hold(-40\,mV)}$).

Determination of adenine nucleotides

The analytical method to determine adenine nucleotides has been reported previously (Hallström *et al.*, 2002). Alterations of this method are in brief: separation was performed on a Hypersil ODS column (5 μm, 250 mm × 4 mm ID) using a Waters™ 717 plus Autosampler (Waters, Milford, MA, U.S.A.), two constaMetric III pumps, a gradient controller (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) and a Waters 969 photodiode array detector. Detector signals (absorbance at 254 nm) were recorded with an AGC Personal Computer. The program Millennium (Waters) was used for data acquisition and analysis. Cardiomyocytes were deproteinized with 250 μl of 0.4 M perchloric acid. After centrifugation (12,000 × *g*), 100 μl of the acid extract were neutralized with 10 μl of 2 M potassium carbonate (4°C). The supernatant (10 μl) obtained after centrifugation was used for HPLC analysis. The pellets of the acid extract were dissolved in 1 ml of 0.1 M sodium hydroxide and further diluted 1 : 10 with physiologic saline for protein determination (BCA Protein Assay, PIERCE, Rockford, IL, U.S.A.). Percentage of living cells was estimated by determining the ratio of rod-shaped to total myocytes. The percentage of rod-shaped myocytes varied between 28 and 32% in different cell preparations. At least 600 myocytes were counted in each preparation.

Statistics

Data are expressed as means ± s.e.m., n = number of cells. Error bars in figures represent s.e.m. Statistical significance was determined by a two-tailed Student's *t*-test or, if more than two conditions were compared, by one-way analysis of variance (ANOVA) with the LSD *post hoc* test. Differences were considered significant at $P<0.05$.

Drugs

Stock solutions of $K_{(ATP)}$ channel blocker glibenclamide and of $K_{(ATP)}$ channel opener pinacidil were prepared daily in DMSO. Both drugs were purchased from Sigma.

NADH (β-nicotinamide adenine dinucleotide, reduced form, disodium salt) was purchased from Roche (Mannheim, Germany), nicotinamide and NAD^+ (β-nicotinamide adenine dinucleotide, oxidized form) from Sigma.

Results

Activation of $I_{K(ATP)}$ by pinacidil

Mean membrane capacitance (C_m) of isolated guinea-pig ventricular myocytes used in this study was 113.6±3.13 pF ($n=102$). Under physiological conditions (4.3 mM ATP in the pipette solution, ATP_i), outward current density of I_{ramp} was 2.19±0.26 pA pF^{-1} at +30 mV ($n=19$). $I_{K(ATP)}$ did not contribute to the basal electrical activity since glibenclamide (50 μM), a $K_{(ATP)}$ channel blocker, did not affect I_{ramp} (data not shown). Figure 1 shows the effect of pinacidil on $I_{hold(-40\,mV)}$ and I_{ramp}. Exposure to 30 μM pinacidil caused $I_{K(ATP)}$ activation, shown as a strong increase in $I_{hold(-40\,mV)}$. Superfusion of the cell with the sulfonylurea glibenclamide (1 μM) completely

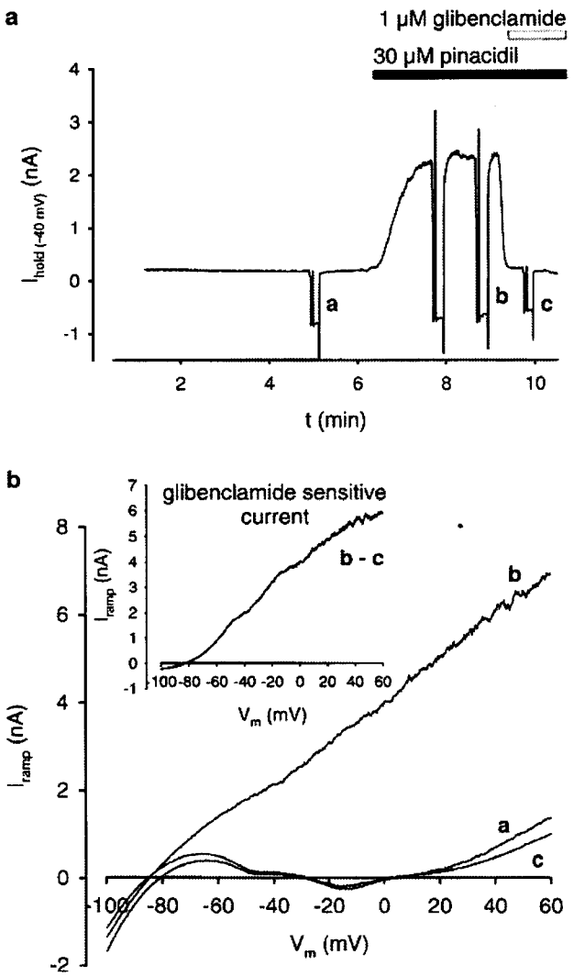

Figure 1 (a) Time course of $I_{K(ATP)}$ activation by pinacidil (30 μM) and subsequent blockade by glibenclamide (1 μM) in a guinea-pig ventricular myocyte. Sharp vertical deviations represent the voltage ramp-elicited current traces. (b) Original current traces, elicited by a 2-s voltage ramp, recorded at different stages of the experimental protocol indicated by letters in (a). The inset shows the glibenclamide-sensitive current, that is $I_{K(ATP)}$, evaluated by digital subtraction.

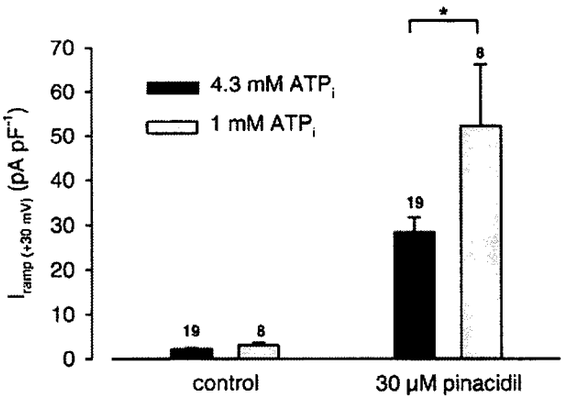

Figure 2 Outward current density (at +30 mV) under control conditions and in response to 30 μM pinacidil at different concentrations of ATP_i (1 and 4.3 mM). Numbers given represent n. *$P<0.05$.

inhibited $I_{K(ATP)}$ almost immediately, and the holding current returned to control level (Figure 1a). Figure 1b shows the original current traces elicited by a voltage ramp applied at different stages of the experimental protocol (indicated by letters in (a)). The current–voltage relation recorded under control conditions revealed the shape typical for ventricular myocytes. During application of pinacidil, a large increase in membrane current was observed. Addition of 1 μM glibenclamide completely reversed this effect. The glibenclamide-sensitive current (inset of Figure 1b) obtained by digital subtraction represents $I_{K(ATP)}$ showing an almost linear current–voltage relation. The reversal potential of about −80 mV indicates high potassium selectivity. Under exposure of 30 μM pinacidil, the outward current density at +30 mV was 28.9±3.38 pA pF^{-1} ($n=19$). After a washout period of 5 min, the current density returned to control values (1.81±0.22 pA pF^{-1}, $n=12$). Figure 2 shows the pinacidil (30 μM)-induced activation of $I_{K(ATP)}$, demonstrated as the increase in I_{ramp} density at +30 mV in the presence of a physiological (4.3 mM) and a low (1 mM) ATP_i concentration. Under control conditions (measured 5 min after rupture of the membrane patch), $I_{ramp(+30\,mV)}$ density was not statistically different at physiological and low ATP_i (2.19±0.26 pA pF^{-1} ($n=19$) *versus* 3.02±0.51 pA pF^{-1} ($n=8$) using 4.3 and 1 mM ATP_i, respectively). However, using 1 mM ATP_i, the pinacidil-induced increase in outward current density was significantly higher compared to 4.3 mM ATP_i ($P<0.05$). The current density was 28.3±3.26 ($n=19$) and 52.2±13.8 pA pF^{-1} ($n=8$) at 4.3 and 1 mM ATP_i, respectively. Thus, the pinacidil-primed $I_{K(ATP)}$ is an indirect indicator of subsarcolemmal ATP concentration.

Effects of incubation with NADH and related compounds

Figure 3 shows the concentration-dependent effect of NADH on $I_{K(ATP)}$ activation by 30 μM pinacidil under physiological conditions (4.3 mM ATP_i). Guinea-pig ventricular myocytes were incubated with different concentrations of NADH (200, 300, 400, 800, 1600 μg ml^{-1} cell culture medium) for 4–6 h before electrophysiological experiments were performed. For

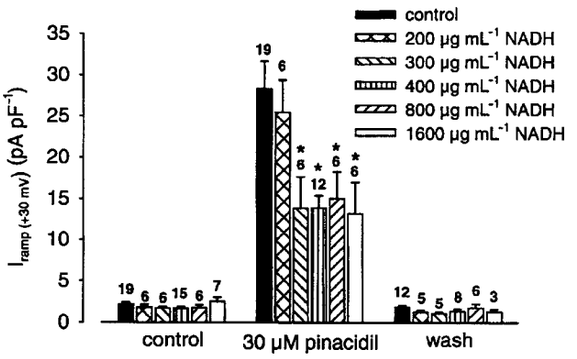

Figure 3 Concentration-dependent effects of NADH incubation on $I_{K(ATP)}$ activation by 30 μM pinacidil. Numbers given represent *n*. *$P<0.05$ compared to control myocytes.

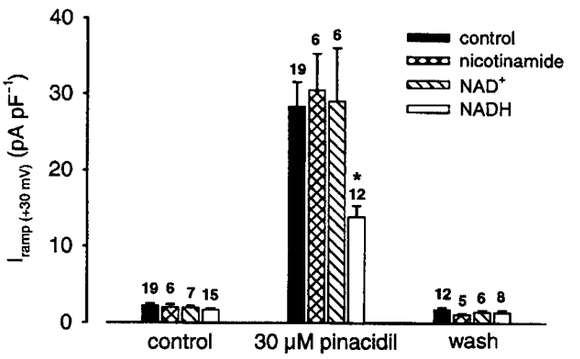

Figure 4 $I_{K(ATP)}$ activation by 30 μM pinacidil in myocytes incubated with 400 μg ml^{-1} NADH and equimolar amounts of nicotinamide and NAD$^+$. Numbers given represent *n*. *$P<0.05$ compared to all groups under 30 μM pinacidil.

control experiments, an identical time schedule was used. Under control conditions, outward current density at +30 mV was not different between control and NADH-incubated cells. The current density was 2.19±0.26 ($n=19$), 1.82±0.33 ($n=6$), 1.72±0.19 ($n=6$), 1.70±0.18 ($n=15$), 1.76±0.36 ($n=6$) and 2.53±0.49 pA pF^{-1} ($n=7$) in control and after incubation with 200, 300, 400, 800 and 1600 μg ml^{-1} NADH, respectively. Incubation of the myocytes with 200 μg ml^{-1} NADH resulted in a reduced $I_{K(ATP)}$ activation by pinacidil compared to control cells, but this effect did not reach statistical significance (28.3±3.26 ($n=19$) *versus* 25.4±3.97 pA pF^{-1} ($n=6$) at +30 mV). However, using 300 μg ml^{-1}, NADH outward current density was significantly reduced (13.8±3.78 pA pF^{-1}, $n=6$; $P<0.05$). A further increase of NADH concentrations did not further diminish the effect of pinacidil on $I_{K(ATP)}$ activation. The outward current density was 13.9±1.49 ($n=12$), 15.0±3.19 ($n=6$) and 13.2±3.74 pA pF^{-1} ($n=6$) in myocytes incubated with 400, 800 and 1600 μg ml^{-1} NADH, respectively. The effect of the $K_{(ATP)}$ channel opener pinacidil could be washed out completely. The outward current density returned almost to the initial value and was 1.81±0.21 ($n=12$), 1.24±0.24 ($n=5$), 1.09±0.14 ($n=5$), 1.37±0.25 ($n=8$), 1.75±0.48 ($n=6$) and 1.29±0.31 pA pF^{-1} ($n=3$) in control and after incubation with 200, 300, 400, 800 and 1600 μg ml^{-1} NADH, respectively.

To investigate whether this decreased activation of $I_{K(ATP)}$ by pinacidil is a specific effect of NADH or can also be caused by related compounds, the results with 400 μg ml^{-1} NADH were compared with equimolar amounts of nicotinamide and NAD$^+$ (Figure 4). Under control conditions, no difference in outward current density was observed (2.19±0.26 ($n=19$), 2.01±0.36 ($n=6$), 1.96±0.20 ($n=7$) and 1.70±0.18 pA pF^{-1} ($n=15$) in control and after incubation with nicotinamide, NAD$^+$ and NADH, respectively). $I_{K(ATP)}$ activation by pinacidil, however, could neither be reduced by nicotinamide nor by NAD$^+$. There was no statistically significant difference between currents in control myocytes and myocytes incubated with nicotinamide or NAD$^+$. Outward current density at +30 mV after addition of 30 μM pinacidil was 28.3±3.26 pA pF^{-1} ($n=19$) in control myocytes, 30.5±4.83 ($n=6$) and 29.0±7.01 pA pF^{-1} ($n=6$) in nicotinamide and NAD$^+$-incubated cells, respectively. After a washout period, the outward current density returned nearly to control values (1.81±0.22 ($n=12$), 1.13±0.15 ($n=5$), 1.44±0.25 ($n=6$) and 1.37±0.25 pA pF^{-1} ($n=8$) in control and after incubation with nicotinamide, NAD$^+$ and NADH, respectively).

Since $I_{K(ATP)}$ serves as an indicator of subsarcolemmal ATP concentration, the most likely explanation for the observed effects is an NADH-induced increase in intracellular ATP content. A direct effect of extracellular NADH on pinacidil-activated $I_{K(ATP)}$ can be excluded. Simultaneous superfusion of myocytes with 400 μg ml^{-1} NADH and 30 μM pinacidil did not change I_{ramp} (data not shown).

Determination of intracellular adenine nucleotides

To confirm an NADH supplementation-induced increase in ATP_i concentration, we measured the intracellular adenine nucleotide content in cardiomyocytes with and without NADH supplementation by HPLC. Figure 5 shows the summarized results. The increase in ATP content in cardiomyocytes after 4 h supplementation with NADH was highly significant (45.59±1.88 nmol mg^{-1} protein of living cells *versus* control, 35.35±2.57 nmol mg^{-1} protein of living cells, $P<0.000005$, $n=7$), whereas ADP and AMP showed no significant alteration compared to control.

Discussion

Almost all of the cardiac ATP is regenerated by respiratory chain-linked phosphorylation, whereby the reduction potential for the respiratory chain is mainly supplied as the reduced coenzyme NADH which is oxidized by complex I of the respiratory chain. The cellular NADH content can be influenced by extracellular supply of metabolic substrates. Recently, Williams *et al.* (2001) showed that because of the presence of glutamate during the isolation procedure, the intracellular glutamate concentration in single isolated rat myocytes is raised; this in turn increased metabolic flux as indicated by a higher NADH/NAD$^+$ ratio and ATP content as well as improved recovery from simulated hypoxia. The NADH/NAD$^+$ ratio can also be increased by the addition of other metabolic substrates like pyruvate (White & Wittenberg,

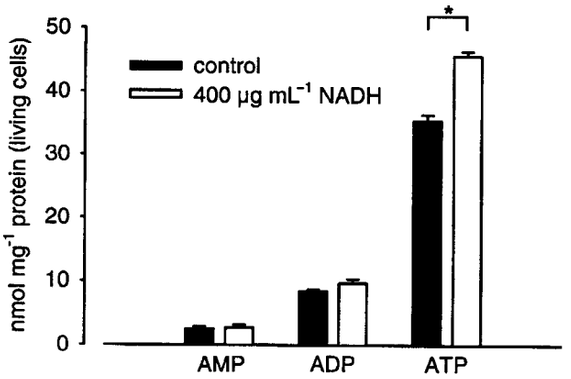

Figure 5 Adenine nucleotide content of cardiomyocytes incubated with 400 μg ml^{-1} NADH for 4 h *versus* control. The values were calculated as 100% living cells. $n=7$ in each group. *$P<0.000005$.

2000; Williams *et al.*, 2001). The activity of cardiac K_{ATP} channels is controlled by a cytosolic ATP pool for which oxidative phosphorylation is the predominat ATP source (Knopp *et al.*, 2001). Since the respiratory chain is fuelled mainly with NADH, it was the aim of this study to investigate, whether NADH supplementation *per se* leads to an improved metabolic state of cardiomyocytes. The pinacidil-primed $I_{K(ATP)}$ was used as sensor of the subsarcolemmal ATP concentration.

Under physiological conditions, $K_{(ATP)}$ channels are predominantly in the closed state because of a strong inhibition of channel activity at an ATP_i concentration in the millimolar range (Noma, 1983; Heidbüchel *et al.*, 1990; Koumi *et al.*, 1997). These data are consistent with our observations in guinea-pig ventricular myocytes revealing no contribution of $I_{K(ATP)}$ to the basal electrical activity, since under physiological conditions the sulfonylurea glibenclamide (50 μM), a $K_{(ATP)}$ channel blocker, did not affect current–voltage relation elicited by a voltage ramp (data not shown).

In the present study, pinacidil, a $K_{(ATP)}$ channel opener (Smallwood & Steinberg, 1988; Arena & Kass, 1989), which is known to increase the open probability of the $K_{(ATP)}$ channel (Isomoto & Kurachi, 1997), activated $I_{K(ATP)}$ showing similar characteristics as described in other studies (Noma & Shibasaki, 1985; Arena & Kass, 1989; Koumi *et al.*, 1997).

Using a physiological (4.3 mM) and a low (1 mM) ATP_i concentration, we could show that pinacidil causes a significantly more pronounced activation of $I_{K(ATP)}$ at a low ATP_i concentration. These results are in line with previous observations reporting the pinacidil action to be dependent on ATP_i with increasing sensitivity to openers at lower ATP_i (Arena & Kass, 1989; Nakayama *et al.*, 1990; Tseng & Hoffman, 1990; Pelzmann *et al.*, 2001). Thus, the pinacidil-primed $I_{K(ATP)}$ serves as an indicator of subsarcolemmal ATP concentration as demonstrated by Sasaki *et al.* (2001).

The results of this study show that supplementation of guinea-pig ventricular myocytes with 300 μg ml^{-1} NADH (4–6 h) causes a significantly reduced $I_{K(ATP)}$ activation by pinacidil compared to control cells, indicating an increased subsarcolemmal ATP concentration. These findings could be confirmed by measuring the intracellular adenine nucleotide content using HPLC, revealing a highly significant increase in ATP content in cardiomyocytes supplemented with NADH, whereas ADP and AMP did not significantly differ from control. Any direct effects of extracellular NADH on $I_{K(ATP)}$ could be excluded. Clinical studies have demonstrated a positive effect of NADH treatment on patients suffering from Morbus Parkinson, chronic fatigue syndrome and depression (Birkmayer & Birkmayer, 1991; Birkmayer *et al.*, 1993; Kuhn *et al.*, 1996; Forsyth *et al.*, 1999). Vrecko *et al.* (1997) showed that NADH supplementation of PC12 cells leads to increased dopamine production being of interest for the treatment of Morbus Parkinson which is characterized by a dopamine deficit. NADH-induced increase of dopamine release could also be shown in rat striatal slices (Pearl *et al.*, 2000). These data indicate that extracellular application of NADH results in cellular functional alterations, whereby the mechanisms are not completely understood. In nigrostriatal dopaminergic terminals release of dopamine is modulated by $K_{(ATP)}$ channels, whereby the extracellular concentration of dopamine is significantly decreased by the PCO cromakalim (Zhu *et al.*, 1999). Thus, modulation of $K_{(ATP)}$ channels obviously plays an important role in dopamine release, whereby a modulation of $K_{(ATP)}$ channel activity by NADH, as described in this work, could be of importance.

In summary, our data show that NADH supplementation, but not of the related compounds nicotinamide and NAD^+, improves the metabolic state of isolated ventricular myocytes indicated by a decreased pinacidil-primed $I_{K(ATP)}$. Measurement of adenine nucleotides confirmed a significant elevation of ATP levels in cardiomyocytes treated with NADH. The mechanism of this increase (elevated NADH/NAD^+ ratio, enhanced mitochondrial ATP production, enhanced intracellular reduction potential or NADH induced enzymatic alterations) is at present under investigation. NADH supplementation could be a useful pharmacological and therapeutical tool producing beneficial effects upon cell metabolism of cardiomyocytes and other high-energy-demanding cells *in vivo*.

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