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NADH -supplementation decreases pinacidil-primed $I_{K(ATP)}$ in ventricular cardiomyocytes via increase of intracellular ATP content

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Abstract

The aim of this study was to investigate the effect of NADH-supplementation on the metabolic condition of isolated guinea pig ventricular cardiomyocytes using the pinacidil-primed $I_{K(ATp)}$ as an indicator of subsarcolemmal ATP concentration. Membrane currents were studied using the patch-clamp technique in the whole-cell recording mode at 36-37°C. Under physiological conditions (4.3 mM ATP in the pipette solution, ATP_j) $I_{K(ATP)}$ did not contribute to basal electrical activity The $K_{(ATP)}$ channel opener pinacidil activated $I_{K(ATP)}$ dependent on $[ATP]_i$ showing a significantly more pronounced activation at lower (1 mM) $[ATP]_j$. Incubation of cardiomyocytes with 300 µg/ml NADH (4-6 h) resulted in a significantly reduced $I_{K(ATp)}$ activation by pinacidil compared to control cells. Equimolar amounts of the related compounds nicotinamide and NAD^+ were not able to achieve a similar effect like NADH. These data show that incubation 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 due to NADH -supplementation. Measurement of adenine nucleotides by HPLC revealed a significant increase in intracellular ATP (NADH supplementation: 45.59 ± 1,88 nmol/mg protein versus control: 35.35 ± 2.57 nmol/mg protein, $P < 0.000005$).

Key words: NADH, $I_{K(ATP)}$, ATP-dependent potassium current, pinacidil, whole-cell-clamp, guinea pig ventricular myocytes.

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 [1-3].

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 cardiac myocyte 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 physiological conditions $K_{(ATP)}$-channels are predominantly closed due to inhibition by intracellular ATP (ATP_i) [4-6]. However, when the ATP_i concentration falls below certain values (like in pathological states such as acute myocardial ischaemia) 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 [7 -10]. Thus, the amount Of $I_{K(ATP)}$ activation induced by these drugs serves as an indicator of the cellular ATP-content [111. Studying the PCO-primed $I_{(KATP)}$ we report that incubation of cardiomyocytes with NADH but not with the related compounds nicotinamide and NAD^+ results in a decrease of $I_{K(ATP)}$ activation by pinacidil consistent with a determined increase of cellular ATP content induced by extracellular application of NADH.

Methods

Myocyte isolation

Guinea pig ventricular myocytes were isolated by Langendorff perfusion using collagenase as described previously [12]. The isolated myocytes were stored in a cell culture medium M 199 (Sigma, St. Louis, MO, USA), supplemented with 5 µg/ml penicillin and 5 IU/ml of streptomycin and were kept in an incubator at 37C. Experiments were performed within 24 h after isolation.

Incubation procedure

The isolated myocytes were incubated with NADH ((ß-nicotinamide adenine dinucleotide, reduced form, disodium salt, Roche, Mannheim, Germany) and the related compounds nicotinamide and NAD^+ (11-nicotinamide adenine dinucleotide, oxidized form, Sigma) in eqimolar amounts 4 to 6 h before electrophysiological parameters were evaluated.

Electrophysiological recordings and data analysis

Membrane currents were recorded using the whole-cell single electrode voltageclamp configuration of the patch-clamp technique [13] using a List L/M-EPC 7 amplifier (List, Darmstadt, Germany) as previously described by Pelzmann and coworkers [10]. Myocytes were placed in an experimental chamber mounted on the stage of an inverted microscope (Axiovert, Zeiss, Oberkochen, Germany) and superfused with standard extracellular solution (composition in mM: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, MgC l_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^O-37^OC with a flow rate of about 1.5 ml/min. When filled with standard internal solution (composition in mM- KCl 110, ATP/K^+ 4.3, $MgCl_2$ 2, CaC l_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 to 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 an IBM compatible computer connected to a D/A and A/D converter (Digidata 1200, Axon Instruments, Foster City, USA). 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 five minutes 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) 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 ($I_{hold(-40\ mv)}$). Stock solutions of $K_{(ATP)}$ channel blocker glibenclamide (Sigma) and of $K_{(ATP)}$ channel opener pinacidil (Sigma) were prepared in DMSO. Averaged data are expressed as means ± SEM, *n* = number of cells. Error bars in figures represent SEM (except in Fig. 5).

Determination of adenine nucleotides

This analytical method has been reported previously. [1]Alterations in brief: Separation was performed on a Hypersil ODS column (5 µm, 250 mm x 4 mm I.D.) using a AS100 HRLC® automatic sampling system (Bio-Rad), a 127 HPLC solvent module and a 168 diode array detector module (Beckman). Detector signals (absorbance at 254 nm) were recorded with an AGC Personal Computer. System Gold™ (Beckman) was used as controller for data requisition and analysis.

Cardiomyocytes were deproteinized with 250µL of 0.4mol/L perchloric acid. After centrifugation (12,000 g) 200 µL of the acid extract were neutralized with 12.5µ L of 2mol/L potassium carbonate (4^{O}c) The supernatant (10µ l) obtained after centrifugation was used for HPLC analysis. The pellets of the acid extract were dissolved in 1 mL of 0. 1 mol/L sodium hydroxide and further diluted 1:10 with physiologic saline for protein determination (BCA Protein Assay, PIERCE).

Statistical significance was determined by a two-tailed Student's *t*-est 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 when $P < 0.05$.

Results and Discussion

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 at +30 mV (n=19). $I_{K(ATP)}$ did not contribute to the basal electrical activity since glibenclamide, a $K_{(ATP)}$ channel blocker did not affect I_{ramp} (data not shown). Under physiological conditions $K_{(ATP)}$ channels are predominantly in the closed state caused by a strong inhibition of channel activity at an ATP_i concentration in the millimolar level as shown by Noma [4]. Similar observations were made in human atrial myocytes [5, 14].

In several studies the $K_{(ATP)}$ channel opener pinacidil was shown to activate $I_{K(ATP)}$ in ventricular cardiomyocytes [7, 15] by increasing the open probability of the $K(ATP)$ channel [16].

Figure 1 shows the effect of pinacidil on $I_{hold(-40\ mv)}$ and I_{ramp} of a representative myocyte. 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 inhibited $I_{K(ATP)}$ almost immediately, and the holding current returned to control level (figure 1 A). Figure 1 B shows the original current traces elicited by a voltage ramp applied at different stages of the experimental protocol as indicated by letters in panel A.

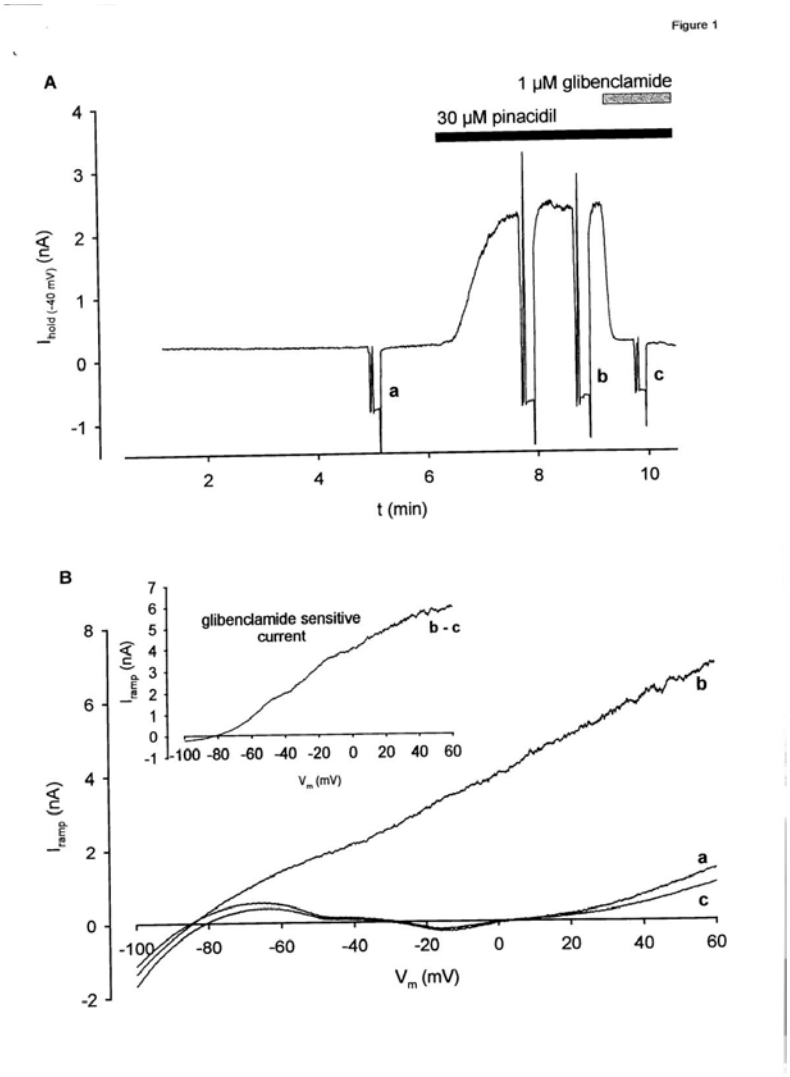


Figure 1: A Time course Of $I_{K(ATP)}$ activation by pinacidil (30 µM) and subsequent blockade by glibenclamide (1 µM) in a representative myocyte. Sharp vertical deviations display the voltage ramp-elicited current traces. **B** Original current traces, elicited by a 2-s voltage ramp, recorded at different stages of the experimental protocol indicated by letters in panel **A.** The inset shows the glibenclamide-sensitive current, i.e. $I_{K(ATP)}$, evaluated by digital subtraction.

The current-voltage relationship recorded under control conditions showed the typical shape for ventricular myocytes. During application of pinacidil a large increase in membrane current could be observed. Addition of 1µM glibenclamide completely reversed this effect, the current returned to almost control values. The glibenclamide-sensitive current (inset of figure 1 B)

obtained by digital subtraction represents $I_{K(ATP)}$ with similar characteristics as described in other studies [7, 14, 17] showing an almost linear current-voltage relationship. A reversal potential of about -80 mV indicates a high selectivity for potassium ions. Under exposure of 30 μM pinacidil the outward current density at +30 mV was 28.9 ± 3.38 pA/pF (n= 19). After a washout period of 5 min the current density returned to control values (1. 81 ± 0.22 pA/pF, 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 five minutes after rupture of the membrane patch) $I_{ramp\ (+3o\ mv)}$ density was not statistically different at physiological and low ATP_i [2.19 ± 0.26 (n=1 9) and 3.02 ± 0.51 (n=8) pA/pF 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 ATPj (P < 0. 05). The current density was 28.3 ± 3.26 (n= 19) and 52.2 ± 13.8 (n=8) pA/pF at 4.3 and 1 mM ATP_i, respectively. These results are in line with previous observations reporting that the action of pinacidil depends on ATPj with increasing sensitivity to openers at lower ATP_i (7-10]. Thus, the pinacidil-primed $I_{K(ATP)}$ serves as an indicator of subsarcolemmal ATP concentration as already convincingly shown by Sasaki and coworkers [11].

Figure 2

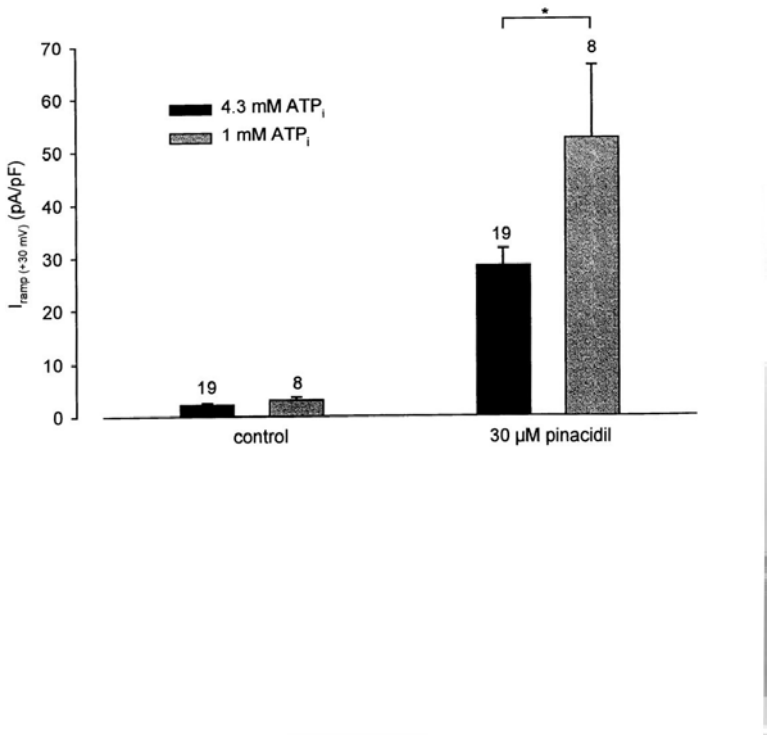


Figure 2: Increase of outward current density at +30 mV by 30 µM pinacidil in response to different concentrations of ATP_i (1 and 4.3 mM). Numbers displayed represent n. * $P < 0.05$.

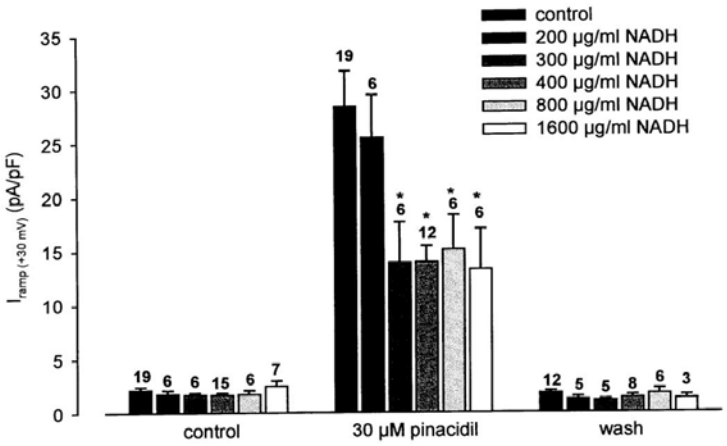
Almost all of the cardiac ATP is regenerated by respiratory chain-linked phosphorylation whereby the energy reaching the respiratory chain is mainly supplied as the reduced coenzyme NADH which is oxidized by complex I of the respiratory chain. The cellular NADH content can be influenced by extracellular supply of metabolic substrates. Recently, Williams et al. (2001) [18] showed that due to the presence of glutamate during isolation procedure the intracellular glutamate concentration in single isolated rat myocytes could be 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. NADH/NAD^+ ratio could also be increased by the addition of other metabolic substrates like pyruvate [18, 19]. The activity of cardiac K_{ATP}

channels is controlled by a cytosolic ATP-pool for which oxidative phosphorylation is the predominat ATP source (20]. 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 using the pinacidil-primed $I_{K(ATP)}$ as a sensor of the 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 cell-culture medium) for 4-6 h before electrophysiological experiments were performed.

Figure 3



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 (n=7) pA/pF in control and after incubation with 200, 300, 400, 800 and 1600 µg/ml NADH, respectively. Incubation of the myocytes with 200 µg/ml NADH resulted in a reduced $I_{K(ATP)}$ activation by pinacidil compared to control cells, but this effect did not reach statistical µ/ml NADH outward current density after application of pinacidil was significantly reduced (13.8 ± 3.78, n=6; P < 0.05). A further increase of NADHconcentration 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 (n=6) pA/pF in myocytes incubated with 400, 800 and 1600 pg/ml 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 (n=3) in control and after incubation with 200, 300, 400, 800 and 1600 µg/ml NADH, respectively.

To confirm the mechanism of NADH supplementation induced decrease of pinacidilprimed $I_{k(ATP)}$ we measured the intracellular adenine nucleotide content in cardiomyocytes with and without NADH supplementation. Figure 5 shows the summarized results. The increase in ATP content of the cardiomyocytes after 4 h supplementation with NADH was highly significant (45.59 ± 1,88 nmol/mg protein versus control, 35.35 ± 2.57 nmol/mg protein, $P < 0.000005$, n=7), whereas ADP and AMP showed no significant alteration compared to control. Further studies will focus on the mechanism of this NADH induced increase of intracellular ATP.

To investigate if 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 NADH were compared with eqimolar amounts of nicotinamide and NAD^+ (figure 4).

Figure 4

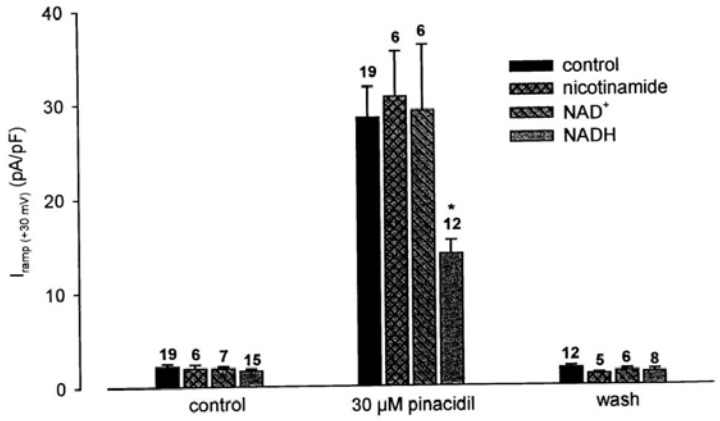


Figure 4: $I_{K(ATP)}$ activation by 30 µM pinacidil in myocytes incubated with 400 µg/ml NADH and equimolar amounts of nicotinamide and NAD^+. Numbers displayed represent *n.* * $P < 0.05$ compared to all groups under 30 µM pinacidil.

Under control conditions there was no difference in outward current density [2.19 0.26 (n=1 9), 2.01 ± 0.36 (n=6), 1.96 ± 0.20 (n=7) and 1.70 ± 0.18 (n=1 5) pA/pF 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^+ to the same extent like by NADH ($P < 0.05$); there was no statistically significant difference between currents in control myocytes and myocytes incubated with nicotinamide or NAD^+. Outward current density at +30mV after addition of 30 pM pinacidil was 28.3 ± 3.26 pA/pF (n=1 9) in control myocytes

and 30.5 ± 4.83 (n=6) and 29.0 ± 7.01 (n=6) pA/pF in nicotinamide and NAD^+ incubated cells, respectively. After a washout period the outward current density returned to almost control values. It was 1.81 ± 0.22 (n=1 2), 1.13 ± 0.15 (n=5), 1.44 ± 0.25 (n=6) and 1.37 ± 0.25 (n=8) in control and after incubation with nicotinamide, NAD+ and NADH, respectively.

Clinical studies have demonstrated a positive effect of NADH treatment on patients suffering from Morbus Parkinson, chronic fatigue syndrome and depression [1 -3]. Vrecko and coworkers [21] 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 [22). 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 was significantly decreased by the PCO cromakalim [23]. 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 for influencing dopamine release.

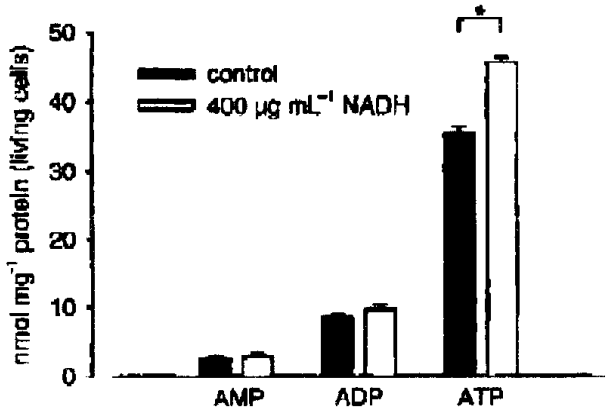


Figure 5 Adenine nucleotide content of cardiomyocytes incubated with 400 µg ml^{-1} NADH for 4 h versus control. The values were calculated as 100% living cells. $n = 7$ in each group. * $P < 0.000005$.

Figure 5: Adenine nucleotide content of cardiomyocytes incubated with 400 µg/mL NADH for 4 h versus control. The values were calculated as 100 % living cells Mean ± SID, n=7 in each group. * $P < 0.000005$.

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.

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