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Differential modulation of cardiac and skeletal muscle ryanodine receptors by NADH

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Abstract The effects of nicotinamide-adenine dinucleotides (NADH, NAD^+, NADPH) on ryanodine receptor channels (RyRs) from cardiac and skeletal muscle were compared in planar bilayers. NADH decreased cardiac RyR activity, which was counteracted by NAD^+. In contrast, NADH and NAD^+ both activated skeletal RyRs. NADPH/$NADP^+$ were without effect on cardiac and skeletal RyRs. In the presence of ATP, NADH inhibition of cardiac RyRs remained. Differently, in the presence of ATP both NADH and NAD^+ were ineffective as skeletal RyR agonists, suggesting interactions with the ATP binding site(s) of the channels. The results suggest that the direct effect of cytosolic NADH is physiologically important for the modulation of cardiac RyRs.

Key words: Sarcoplasmic reticulum; Ryanodine receptor; Ca^{2+} release channel; NADH; Redox potential

1. Introduction

Muscle contraction during excitation–contraction (EC) coupling is triggered by intracellular Ca^{2+} release from sarcoplasmic reticulum (SR) Ca^{2+} stores, which is mediated by the activation of Ca^{2+} release channels or ryanodine receptors (RyRs). In mammalian muscles, three isoforms of RyRs have been identified. The RyR1 isoform is dominant in skeletal muscles, whereas RyR2 represents ~99% of cardiac RyRs. The RyR3 isoform is found only at low levels in some skeletal muscles [1,2]. The gating properties of RyRs are controlled by a variety of cytosolic factors, which suggests a complex regulation of these channels [1,2].

Among those factors the cytosolic redox potential seems to play a role in RyR modulation [1–7]. The NADH/NAD^+ levels are one of the important factors that control the redox state of the cytosol. Certain metabolic conditions can significantly alter NADH/NAD^+ levels. For example, the cytosolic NADH accumulation in ischemic myocardium results from impairment of mitochondrial substrate shuttle systems and from a decreased rate of cytosolic metabolism associated with increased levels of intracellular lactate [8]. It is accepted that ischemia leads to impairment of EC coupling and to the generation of spontaneous SR Ca^{2+} oscillations [9], indicative that a change in RyR function may be involved. It was found that two key enzymes for the regulation of cytosolic NADH/NAD^+ levels (glyceraldehyde 3-phosphate dehydrogenase, GAPDH, and lactate dehydrogenase, LDH) are associated with SR membranes [10,11]. Changes in activity of these enzymes may lead to significant changes of local NADH/NAD^+ levels in close proximity to RyRs, which could affect the function of these channels. There is indeed some evidence that NADH and NAD^+ may modulate RyRs [12–14].

The aim of the present study was to investigate a possible role of the cytosolic NADH/NAD^+ levels for the modulation of SR Ca^{2+} release. We have determined the effects of NADH and NAD^+ on cardiac and skeletal muscle RyR channels incorporated in lipid bilayer. Our results suggest a novel direct effect of cytosolic NADH which is physiologically important for the modulation of cardiac RyRs.

2. Materials and methods

All chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified. Rat ventricular and rabbit skeletal muscle SR vesicles were obtained as described previously [15,16]. Planar lipid bilayers were formed from a lipid mixture (Avanti Polar Lipids, Alabaster, AL, USA) containing phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (ratio 5:4:1) which was dissolved in *n*-decane to a final lipid concentration of 45 mg/ml. SR vesicles were added to the *cis*-chamber, which corresponded to the cytosolic side of the RyR channel. The *trans*-chamber (luminal side of RyR) was connected to the virtual ground of the amplifier.

Fusion of SR vesicles and RyR incorporation into bilayers was induced with the *cis*- and *trans*-chambers containing solutions of the following composition (in mM): $CsCH_3SO_3$ 400 (*cis*) and 40 (*trans*); $CaCl_2$ 0.1; HEPES 20; pH 7.2 (CsOH). After channel incorporation, *trans*[$CsCH_3SO_3$] was increased to 400 mM. Free [Ca^{2+}] in the *cis*-chamber was reduced to various levels by addition of EGTA. Free [Ca^{2+}] in the experimental solutions was verified with a Ca^{2+}-sensitive mini-electrode [17]. Concentrations of compounds used in this study did not change the free [Ca^{2+}] in the experimental solution. Single channel currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). All recordings were made at a holding potential of −20 mV. Currents were filtered at 1 kHz and digitized at 5 kHz. Experiments were carried out at room temperature (22–24°C). The mean open probabilities (P_o) of the channels were used as an index of activity. P_o values were calculated from the 50% threshold analysis. In multiple channel experiments, the global open probability of all channels (nP_o) was calculated. For simplest comparison with single channel experiments, we show nP_o/x values, where x represents the number of current levels observed. Data are presented as mean ± S.E.M. of n bilayers. Statistical comparisons between groups were performed with Student's t-test. $P \leq 0.05$ was considered statistically significant.

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3. Results

We studied the effects of NADH and NAD^+ on the activity of cardiac RyRs incorporated into planar lipid bilayers. Fig. 1A illustrates the inhibitory effects of NADH (0.5 and 2 mM; added to the cytosolic side of the channel) on cardiac RyR channel currents and P_o. For cardiac RyRs in 3 μM *cis*[Ca^{2+}], P_o decreased from 0.123 ± 0.018 ($n = 8$) under control conditions to 0.089 ± 0.024 ($n = 8$) in the presence of 0.5 mM NADH (inhibition of $29 \pm 10\%$, $P < 0.05$). At 2 mM NADH, P_o decreased to 0.019 ± 0.008 (inhibition of $84 \pm 7\%$, $n = 8$, $P < 0.002$). NADH added to the luminal side did not produce any significant effect on RyR2 channel activity (data not shown). Single channel current amplitudes remained constant during the time of the experiment indicating no effects on the properties of channel conduction.

The activity of cardiac RyRs increased when NAD^+ (the oxidized form of NADH) was added to the cytosolic side of the channels (Fig. 1B). P_o increased from 0.108 ± 0.024 ($n = 7$) under control conditions (*cis*[Ca^{2+}] 3 μM) to 0.142 ± 0.028 ($n = 7$) in the presence of 2 mM NAD^+ (activation by $32 \pm 9\%$, $P < 0.05$). By raising NAD^+ to 4 mM, P_o increased further to 0.219 ± 0.024 ($n = 7$), i.e. reflecting an activation by $107 \pm 20\%$ ($n = 7$, $P < 0.05$). Again, luminal NAD^+ was without effect on P_o. Furthermore, NAD^+ (luminal or cytosolic application) did not affect the conductive properties of the cardiac RyR2.

The finding that NADH and NAD^+, compounds with a similar chemical structure, produced opposite effects on cardiac RyR channels prompted us to test whether the inhibitory effects of NADH can be restored by NAD^+. As illustrated in Fig. 1C, inhibition of cardiac RyRs by NADH (2 mM, cytosolic side) was indeed counteracted by subsequent addition of NAD^+ (2 mM). In the presence of NADH, NAD^+ (2 mM) increased the P_o from 0.009 ± 0.004 to 0.092 ± 0.026 ($n = 4$), which was not significantly different from control level (0.114 ± 0.027, $n = 4$). This effect of NAD^+ did not appear to be related to an independent stimulation of the channel activity, because in control condition NAD^+ (2 mM) had a significantly smaller effect on the RyRs.

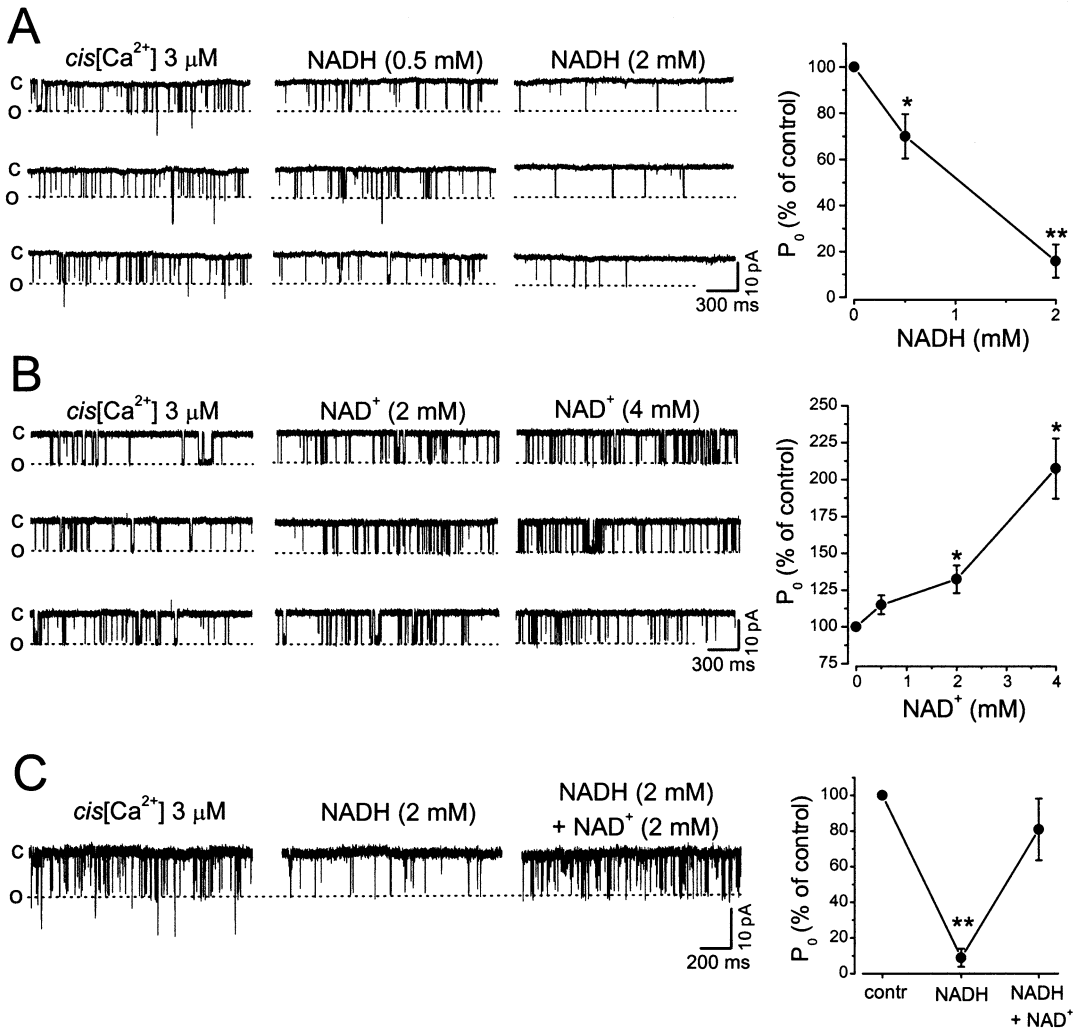

Fig. 1. Effects of NADH and NAD^+ on cardiac RyRs incorporated into planar lipid bilayers. A: (Left) RyR currents in control condition and in the presence of 0.5 and 2 mM NADH. (Right) P_o of cardiac RyRs vs. [NADH]. B: (Left) Cardiac RyR currents in control condition and after addition of 2 and 4 mM NAD^+. (Right) P_o of cardiac RyRs vs. [NAD^+]. C: (Left) Cardiac RyR currents in control condition, after addition of 2 mM NADH and subsequent addition of 2 mM NAD^+. (Right) P_o changes produced by NADH and by subsequent addition of NAD^+. All recordings were made at a holding potential of −20 mV and in the presence of 3 μM *cis*[Ca^{2+}]. The dotted line indicated the open channel level. *Statistically different at $P < 0.05$ and **statistically different at $P < 0.002$.

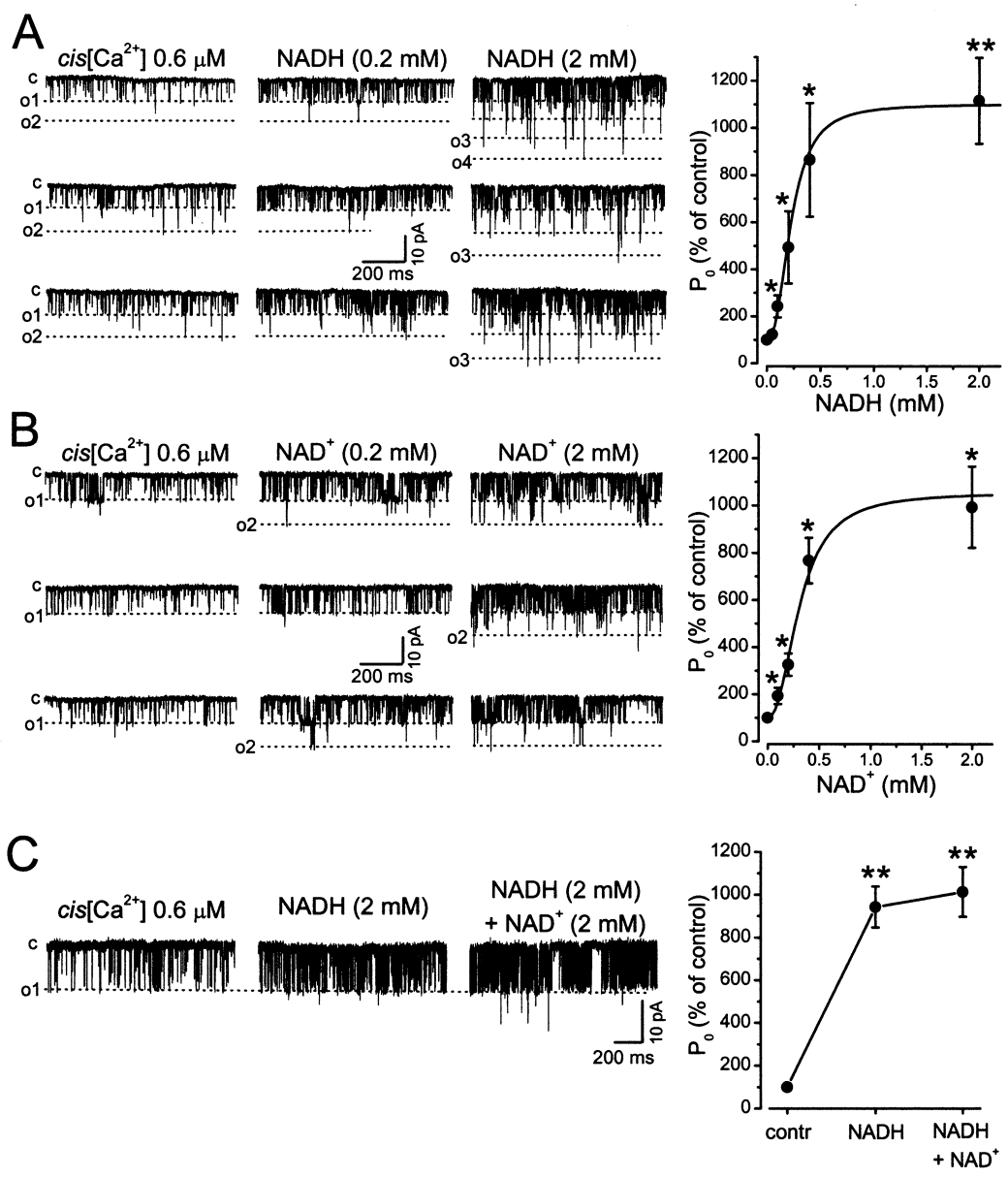

Fig. 2. Effects of NADH and NAD^+ on skeletal RyR incorporated into planar lipid bilayers. A: (Left) RyR currents in control condition and in the presence of 0.2 and 2 mM NADH. (Right) P_o of skeletal RyRs vs. [NADH]. B: (Left) Skeletal RyR currents in control condition and after addition of 0.2 and 2 mM NAD^+. (Right) P_o of skeletal RyRs vs. [NAD^+]. C: (Left) Skeletal RyR currents in control condition, after addition of 2 mM NADH and subsequent addition of 2 mM NAD^+. (Right) Changes of P_o produced by NADH and by subsequent addition of NAD^+. All recordings were made at a holding potential of −20 mV and in the presence of 0.6 μM *cis*[Ca^{2+}]. The dotted line indicates the open channel level. *Statistically different at $P<0.05$ and **statistically different at $P<0.002$.

The cytosolic redox agent NADPH was also tested. We found that NADPH did not inhibit cardiac RyR activity. In four experiments P_o of RyRs (*cis*[Ca^{2+}] = 3 μM) was 0.126 ± 0.024 in control conditions and 0.161 ± 0.032 in 2 mM NADPH ($n=4$). Furthermore, NADPH did not interfere with the inhibitory effects of NADH (results not shown).

Since SR Ca^{2+} release channels in heart and skeletal muscle appear to be subject to different modes of regulation and modulation [1,2], we also tested NADH and NAD^+ effects on skeletal RyR channels (Fig. 2). At variance with cardiac channels (Fig. 1), we found that both NADH (Fig. 2A) and NAD^+ (Fig. 2B) activated skeletal RyRs. As shown in Fig. 2A for skeletal RyRs (*cis*[Ca^{2+}] = 0.6 μM), 0.1 mM NADH activated the channels. With 0.1 mM NADH P_o increased by $142\pm47\%$ ($n=5$, $P<0.05$). Maximal effects were observed with 2 mM NADH, where P_o increased from 0.011 ± 0.005 (control, $n=5$) to 0.119 ± 0.023 ($n=5$), which corresponds to an activation by $1015\pm182\%$ ($n=5$, $P<0.002$). The EC_{50} value for RyR channel activation by NADH was 0.23 mM.

Similar to NADH, NAD^+ also increased the activity of skeletal RyR channels (Fig. 2B). The EC_{50} value for NAD^+ was 0.31 mM and the maximal effect was reached at 2 mM NAD^+. P_o increased from 0.012 ± 0.007 ($n=4$) under control conditions to 0.116 ± 0.024 ($n=4$), reflecting an activation by $893\pm172\%$ ($n=4$, $P<0.05$).

Thus, NADH and NAD^+ had similar effects on skeletal

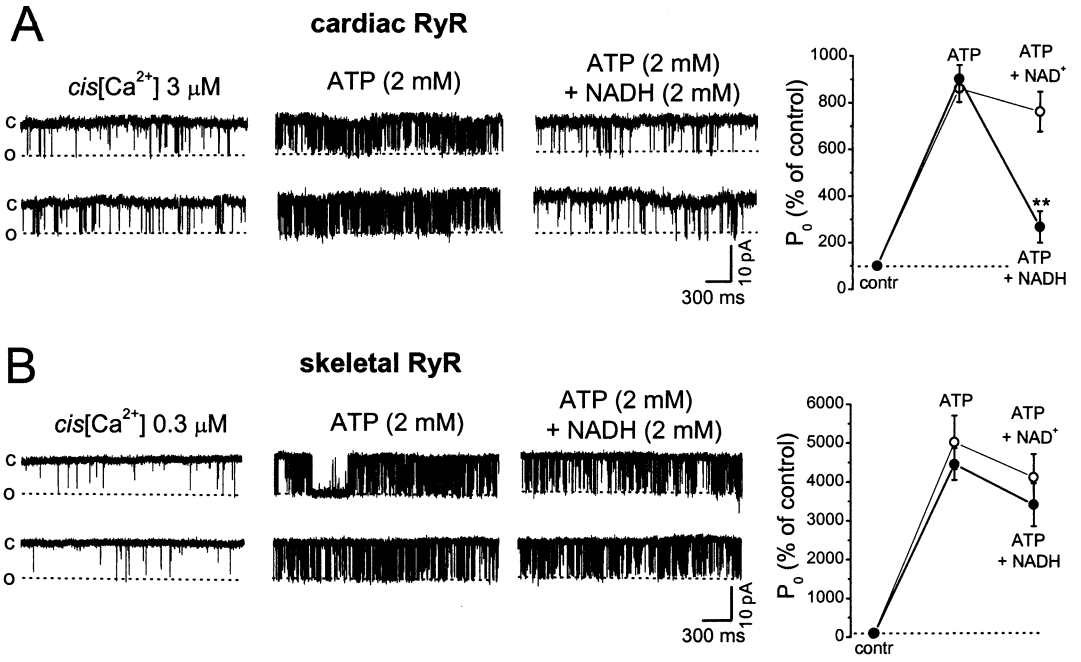

Fig. 3. Effects of NADH and NAD^+ on cardiac and skeletal RyRs activated by ATP. A: (Left) Cardiac RyR currents in control condition (3 μM *cis*[Ca^{2+}]), in the presence of 2 mM ATP and subsequent addition of 2 mM NADH. (Right) Changes in P_o of cardiac RyRs induced by addition of ATP and by subsequent addition of NADH (filled symbols) or NAD^+ (open symbols). B: (Left) Skeletal RyR currents in control condition (0.3 μM *cis*[Ca^{2+}]), in the presence of 2 mM ATP and subsequent addition of 2 mM NADH. (Right) Changes of P_o produced by ATP and by subsequent addition of NADH (filled symbols) or NAD^+ (open symbols). All recordings were made at a holding potential of −20 mV. The dotted line indicated the open channel level. **Statistically different at $P < 0.002$ compared with ATP alone.

muscle RyRs. As expected for molecules interacting with the same binding site, we found that these compounds did not potentiate each other (Fig. 2C). As illustrated in Fig. 2C, NADH (2 mM) increased P_o of RyRs by 843 ± 96% ($n = 3$). Subsequent addition of NAD^+ (2 mM) did not produce a significant further increase in P_o (914 ± 117%, $n = 3$) of the skeletal RyR channels. Vice versa, the P_o of the channels activated by NAD^+ (2 mM) did not significantly change by addition of NADH (2 mM; data not shown).

Similar to cardiac RyRs, we found that NADPH had little or no effect on the activity of skeletal RyRs and did not compete with the activating effects of NAD^+ and NADH.

There is similarity in structure between NAD^+ (or NADH) and ATP, which is an activator of RyRs [12,13]. It is then possible that the effects observed with cardiac and skeletal RyR channels are generated, at least in part, by a distinct interaction of NAD^+ and NADH with the adenine nucleotide binding site(s). If the inhibition of cardiac RyRs by NADH were related to these sites, then ATP (more selective agonist) would partially diminish the NADH/NAD^+ effects. As shown in Fig. 3A we found that ATP (2 mM) significantly activated cardiac RyRs. P_o increased from 0.098 ± 0.013 to 0.793 ± 0.068 ($n = 3$). Nevertheless, NADH (2 mM) markedly decreased P_o values to 0.165 ± 0.037 ($n = 3$), which represents an inhibition of 80 ± 11% (Fig. 3A). This level of inhibition of RyR activity by NADH in ATP-containing solutions was not significantly different from that observed without ATP (see above). Thus, inhibition of the cardiac RyR channel by NADH may involve sites different from adenine nucleotide binding sites of RyRs. Interestingly, for cardiac RyRs exposed to ATP (2 mM), which elevated P_o to 0.813 ± 0.059 ($n = 3$), the subsequent addition of NAD^+ (2 mM) did not further increase channel activity (P_o was 0.719 ± 0.067, $n = 3$). These data, on the other hand, indicate that NAD^+ effects may be related to an agonistic effect on adenine nucleotide binding sites.

Skeletal RyRs display a higher sensitivity to ATP [1,2]. Thus, 2 mM ATP would have maximal effects on the ATP binding sites. As expected for a compound affecting nucleotide binding sites, we found that both NADH and NAD^+ are without effects on the activity of skeletal RyRs incubated with 2 mM ATP. Fig. 3B shows that ATP (2 mM) increased P_o from 0.021 ± 0.008 to 0.891 ± 0.079 ($n = 3$). For these almost fully activated skeletal RyR channels, the subsequent addition of NADH (2 mM) did not significantly change the channel activity (P_o was 0.682 ± 0.092, $n = 3$). As shown on the right of Fig. 3B, NAD^+ had similar effects. These data indicate that the effects of NAD^+ and NADH on skeletal RyRs may be related to partial agonistic effects on the adenine nucleotide binding sites that are competed by ATP.

4. Discussion

Our results show that cytosolic NADH has differential direct effects on cardiac and skeletal RyRs. NADH inhibited cardiac RyR channels and activated skeletal RyRs. Differently, NAD^+ had activating effects on both cardiac and skeletal RyRs. Our results also suggest that only direct inhibition of cardiac RyRs by NADH may have physiological relevance as it remained in the presence of the physiological agonist ATP. Differently, the activating effects of NADH and NAD^+ on skeletal RyRs may just represent interactions with nucleotide binding sites and these effects would be effectively competed by physiological levels of ATP.

There is evidence in the literature that NADH and NAD^+ may affect cardiac and skeletal RyR nucleotide binding sites [12–14]. This is the first report, however, on the inhibitory effect of NADH on cardiac RyRs that appears to involve sites that are not competed by ATP. For skeletal muscle it was reported that RyR1 has an oxidoreductase-like domain that could function as a redox sensor [14]. Our results from skeletal RyRs, however, suggest that the changes of the cytosolic NADH/NAD^+ levels would have little or no effect on the channel when ATP is present. Yet, NADH may bind to similar sites on the cardiac RyR that would affect channel function. Since we utilized RyR channels from native membranes, the observed inhibitory NADH effect may result from a direct interaction of NADH with cardiac RyRs or it could be mediated through ancillary SR proteins that are incorporated into the bilayers together with the RyR channels. Thus, the exact site of the inhibitory NADH action remains to be determined.

We also found that these inhibitory sites are relatively specific for NADH. Interestingly, a similar agent such as NADPH, which is responsible for generating redox potentials directly in the cytosol, did not affect channel activity or compete with the NADH effects. The only structural difference between NADH and NADPH is that the latter compound has a phosphate group esterified to the 2-position of the ribose, which would suggest that this group might impede the interaction with both inhibitory and activating sites on the RyR.

In heart, NADH levels may vary according with levels of hypoxia or lactate accumulation [8]. For this tissue, which usually works in a constant aerobic environment, changes of NADH levels may be indicative of ischemic or hypoxic stress. From our data it is apparent that NADH accumulation would directly affect the modulation of cardiac RyRs, resulting in an inhibition of SR Ca^{2+} release. In contrast, this NADH effect was absent for skeletal RyR channels. Accordingly, skeletal muscle can physiologically work under various levels of oxygenation and contains fibers (fast twitch muscle) which depend mainly on anaerobic glycolysis. Therefore, they experience changing levels of NADH [18] under physiological conditions.

The results presented here do not negate the importance of redox processes for the modulation of skeletal RyR activity. Our experiments utilized an in vitro system where many organelles and cytosolic factors are absent. In cells, changes in NADH levels may have a number of 'indirect effects' that could affect RyR channels. For example, a recent report provides evidence that skeletal muscle SR contains an NADH-dependent oxidase that generates superoxide (O_2^-.), which in turn stimulates RyRs [19]. Additional mechanisms of cytosolic redox modulation of cardiac and skeletal RyRs exist. There is evidence that various oxidants and reducing agents impact single RyR channel function [3–7], and binding of the RyR regulatory protein calmodulin [20]. It is also known that nitric oxide (NO) may modulate RyR channels via *S*-nitrosylation of cysteine residues [21,22].

It becomes increasingly clear that redox modulation of single RyR channels may represent a quite complex mechanism. Nonetheless, our results suggest a novel mechanism of direct regulation of cardiac RyR function by cytosolic NADH levels.

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