NADH Oxidase Activity of Rat Cardiac Sarcoplasmic Reticulum Regulates Calcium-Induced Calcium Release

Gennady Cherednichenko, Aleksey V. Zima, Wei Feng, Saul Schaefer, Lothar A. Blatter, Isaac N. Pessah

Abstract—NADH and Ca\textsuperscript{2+} have important regulatory functions in cardiomyocytes related to excitation-contraction coupling and ATP production. To elucidate elements of these functions, we examined the effect of NADH on sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release and the mechanisms of this regulation. Physiological concentrations of cytosolic NADH inhibited ryano dine receptor type 2 (RyR2)–mediated Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) from SR membranes (IC\textsubscript{50}=120 μmol/L) and significantly lowered single channel open probability. In permeabilized single ventricular cardiomyocytes, NADH significantly inhibited the amplitude and frequency of spontaneous Ca\textsuperscript{2+} release. Blockers of electron transport prevented the inhibitory effect of NADH on CICR in isolated membranes and permeabilized cells, as well as on the activity of RyR2 channels reconstituted in lipid bilayer. An endogenous NADH oxidase activity from rat heart copurified with SR enriched with RyR2. A significant contribution by mitochondria was excluded as NADH oxidation by SR exhibited ≥9-fold higher catalytic activity (8.8 μmol/mg protein per minute) in the absence of exogenous mitochondrial complex (MC) I (ubiquinone) or MC III (cytochrome c) electron acceptors, but was inhibited by rotenone and pyridaben (IC\textsubscript{50}=2 to 3 nmol/mL), antimycin A (IC\textsubscript{50}=13 nmol/mL), and diphenyleneiodonium (IC\textsubscript{50}=28 μmol/mL). Cardiac junctional SR treated with [\textsuperscript{3}H](trifluoromethyl)diazirinyl-pyridaben specifically labeled a single 23-kDa PSST-like protein. These data indicate that NADH oxidation is tightly linked to, and essential for, negative regulation of the RyR2 complex and is a likely component of an important physiological negative-feedback mechanism coupling SR Ca\textsuperscript{2+} fluxes and mitochondrial energy production. (Circ Res. 2004;94:1001–1009.)

Key Words: ryanodine receptors \textbullet\ cardiac SR NADH oxidase \textbullet\ rotenone

In cardiac muscle, cytosolic [Ca\textsuperscript{2+}] is an important dynamic control signal for stimulating the reduction of mitochondrial NAD\textsuperscript{+} to NADH,\textsuperscript{1,2} with [NADH] critical to the control of the oxidative phosphorylation rate and cellular energetic state.\textsuperscript{3–4} An increase in cytoplasmic [Ca\textsuperscript{2+}] activates myofilaments, thereby increasing ATP consumption rates, followed by activation of Ca\textsuperscript{2+}-dependent mitochondrial dehydrogenases that enhance electron transport and increase ATP production.\textsuperscript{5–9} The ability of cytoplasmic Ca\textsuperscript{2+} signals to regulate the activity of mitochondrial enzymes appears to represent a fundamental means of providing feed-forward control of cellular energetic state (ie, cellular [ATP]/[ADP]).\textsuperscript{2,10}

In cardiac ventricular myocytes, beat-to-beat changes in cytoplasmic Ca\textsuperscript{2+} are driven by excitation-contraction (EC) coupling. Electrical depolarization of the transverse (T) tubule membrane activates L-type voltage-gated Ca\textsuperscript{2+} channels that enhance inward flux of Ca\textsuperscript{2+} through \alpha\textsubscript{C}-DHPR and thereby trigger release of Ca\textsuperscript{2+} from SR stores by a mechanism referred to as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR).\textsuperscript{10–13} This involves activation of ryanodine receptor type 2 (RyR2), a multimeric channel complex of ≥2.5 mDa. RyR2, along with its genetic isoforms RyR1 and RyR3, are broadly expressed in muscle and nonmuscle cells such as neurons and cells of the peripheral immune system.\textsuperscript{14,15} The ratio of GSH/GSSG tightly influences RyR1 and RyR2 activity and SR Ca\textsuperscript{2+} release, indicating the presence of a redox-sensing function.\textsuperscript{16–19} This coupling has physiological significance because the redox status within localized regions of cytosol could represent a feedback-control mechanism linking CICR with cellular energy demands and regulation of ATP production in mitochondria. In addition to the GSH/GSSG couple, regulation of RyR2 from cardiac muscle by NADH/NAD\textsuperscript{+} has recently been reported\textsuperscript{20}; however, the underlying mechanisms of this regulation are not known.

Materials and Methods

Membrane Vesicles

Cardiac and skeletal muscle SR membrane fractions enriched in terminal cisternae (junctional SR) were isolated from Sprague-
Dawley rats as previously described. Membrane preparations enriched in mitochondrial particles were obtained from cardiac tissue according to as described in Materials and Methods. After 1-minute incubation of Ca\(^{2+}\)-loaded SR vesicles with 0.1 to 5 mmol/L NADH, Ca\(^{2+}\) release was induced by bolus addition of Ca\(^{2+}\). Ca\(^{2+}\) release rate in the absence of NADH corresponded to 28.6±3.1 nmol Ca\(^{2+}\)/mg protein per minute (mean±SE of n=8 determinations). B, Electron transfer inhibitors rotenone (Rot; 20 nmol/L), pyridaben (Pyr; 20 nmol/L), and antimycin A (Ant A; 50 nmol/L) prevent NADH oxidase–mediated suppression of CICR in cardiac SR. Each inhibitor was added to Ca\(^{2+}\)-loaded SR vesicles immediately before the addition of 5 mmol/L NADH. Data represent the mean±SE of n=8. *P<0.0002 vs control.

**Results and Discussion**

**NADH Oxidase of Cardiac SR Mediates Suppression of CICR and RyR2 Gating**

Cardiac SR vesicles were loaded with sequential addition of Ca\(^{2+}\) in the presence of ATP. Once the loading phase was complete, extraskeletal Ca\(^{2+}\) was elevated to 50 μmol/L in the presence of 1 μmol/L of the SERCA pump inhibitor thapsigargin (to prevent Ca\(^{2+}\) reuptake). This experimental approach resulted in net Ca\(^{2+}\) efflux from vesicles that had two pharmacologically distinct components. One component was inhibited by either micromolar ruthenium red (RuRed) or ryanodine and defined as CICR mediated by RyR2 activation. Inclusion of NADH (0.1 to 5 mmol/L) dose-dependently decreased the initial rate of CICR with half-maximal suppression at 120±14 μmol/L of NADH (Figure 1A). NADH could fully block the RuRed- and ryanodine-sensitive component of efflux, but did not inhibit the “leak” component of efflux. A possible relationship between oxidation of NADH and suppression of CICR was examined with SR vesicles. Rotenone (20 nmol/L), pyridaben (20 nmol/L), or antimycin A (50 nmol/L), added to the SR suspension immediately before introducing 5nmol/L NADH, essentially eliminated its inhibitory actions on CICR (Figure 1B). In separate experiments, each inhibitor added in the absence of NADH had no direct effect on CICR rate. Thus, NADH oxidation by cardiac SR was required for negative modulation of CICR.
Although addition of rotenone (20 nmol/L) to the cis side of the BLM had little influence on $P_o$, it completely relieved the inhibitory influence of subsequent addition of 2 mmol/L NADH. C, Summary data of $P_o$ under the various experimental conditions used. Data represent the mean $\pm$ SE for $n=5$ (Rot), and $n=10$ (NADH), $n=5$ (Rot+NADH) in the presence of 3 $\mu$mol/L cis [Ca$^{2+}$].

The direct influence of NADH on the gating properties of RyR2 was studied with single channels reconstituted in BLM. Figure 2A shows several seconds of active RyR2 channel gating before and after addition of 2 mmol/L NADH on the cis side of the BLM. In the presence of 3 $\mu$mol/L Ca$^{2+}$ cis, the mean channel $P_o$ was $0.121 \pm 0.013$ (n=15). On introducing 2 mmol/L NADH into the cis side of the BLM chamber, a concentration expected to fully saturate the NADH oxidase, the $P_o$ rapidly decreased $\approx 6$ fold $(mean P_o=0.0196 \pm 0.0097; n=10)$ (Figures 2A and 2C). In separate experiments, channel behavior was assessed in the presence of more stimulatory $10\mu$mol/L Ca$^{2+}$ cis; addition of NADH rapidly decreased $P_o$ 30-fold (from $P_o=0.606$ to $P_o=0.019; n=7$). The inhibitory actions of NADH on single channel gating were fully reversible on perfusion of the cis chamber (not shown). Although addition of rotenone (20 $\mu$mol/L) to the cis chamber of the BLM had little influence on $P_o$, it completely eliminated the inhibitory action of NADH (Figures 2B and 2C).

These results strongly suggest that NADH oxidation is an essential step for inhibition of RyR2 at the level of the single channel and CICR. Thus an SR NADH oxidase is closely associated with RyR2 and is likely to represent a physiological negative modulator of RyR2 and CICR in cardiac muscle.20 Interestingly, in the presence of ATP, NADH maintained its inhibitory activity toward RyR2 but had no effect on RyR1 channel gating activity. NAD$^+$ (2 mmol/L), a product of NADH oxidation, had a small stimulatory influence on CICR (125% of control CICR). Moreover, the inhibitory action of NADH on single RyR2 channels was counteracted by NAD$^+$.20 Therefore, CICR from cardiac SR, but not skeletal SR, was responsive to both reduced and oxidized forms and together the ratio of NADH/NAD$^+$ could tightly regulate the rate of Ca$^{2+}$ release from cardiac SR.

**NADH Inhibition of Ca$^{2+}$ Release in Permeabilized Cardiomyocytes**

Permeabilized cardiomyocytes were placed in an intracellular solution containing 200 $\mu$mol/L [Ca$^{2+}$], which produced spontaneous Ca$^{2+}$ release from SR that propagated through the entire cell as repetitive Ca$^{2+}$ waves having constant frequency of $\approx 0.3$ Hz. Figure 3A shows representative line-scan images of Ca$^{2+}$ waves (top panel) and selected plots of $F/F_0$ changes (bottom panel) under control condition, after addition of NADH, and after the subsequent application of NAD$^+$. The influence of NADH (2 mmol/L) and NAD$^+$ (2 mmol/L) on average amplitude, frequency, and propagation velocity of Ca$^{2+}$ waves are summarized in Figure 3B. Addition of NADH to the intracellular solution resulted in significant decrease in the amplitude (defined as $\Delta F/F_0$) as well as the frequency of Ca$^{2+}$ waves. The effects of NADH were associated with a fragmentation of Ca$^{2+}$ waves (Figure 3A, second image) and a reduction of wave propagation velocity by $\approx 25\%$ (from $95.2 \pm 6.1$ to $62.8 \pm 5.5\mu m/s, n=12; P<0.05$). NADH application suppressed Ca$^{2+}$ wave amplitude by 56% (from $2.7 \pm 0.1$ to $1.2 \pm 0.2; n=15; P<0.01$) and decreased the frequency by 32% (from $0.31 \pm 0.03$ to $0.21 \pm 0.04$ Hz; $n=15; P<0.01$). The subsequent addition of 2 mmol/L of NAD$^+$ completely restored the parameters of the Ca$^{2+}$ waves to control level. However, NAD$^+$ itself did not produce any significant effects on SR Ca$^{2+}$ release (not
Figure 3. NADH negatively modulates spontaneous Ca\(^{2+}\) waves in permeabilized ventricular myocytes. Membrane permeabilization as described in Materials and Methods resulted in spontaneous Ca\(^{2+}\) release from SR that propagated as Ca\(^{2+}\) waves with a frequency of \(-0.3\) Hz. A, Representative line-scan images (top) and selected plots of F/F\(_0\) changes (bottom) under control condition, after addition of NADH, and after the subsequent application of NAD\(^{+}\). F/F\(_0\) plots were obtained by averaging fluo-3 fluorescence from 6-\(\mu\)m-wide region marked by a white box. B, Summary data of the influence of NADH and NAD\(^{+}\) on average amplitude, frequency, and velocity of Ca\(^{2+}\) waves (mean\(\pm\)SE of n=12 to 15 myocytes). C, Rotenone prevents the inhibitory actions of NADH (2 mmol/L) on amplitude of spontaneous events and reduces its impact on their frequency (see text for details). *P<0.05 vs rotenone alone.
NADPH (2 mmol/L) did not significantly alter the amplitude of spontaneous events (not shown). Therefore NADH abolished the inhibitory effect of NADH rather than produced independent additive stimulation of Ca^{2+} release under these experimental conditions. These actions of NADH were specific to this nucleotide because NADPH (2 mmol/L) did not significantly alter the amplitude of spontaneous events (not shown).

Increase of the temperature to 35°C resulted in a significantly higher frequency of Ca^{2+} waves than at room temperature. The Ca^{2+} waves became more irregular, and multifocal Ca^{2+} release was observed, which made it difficult to derive quantitative parameters of wave frequency and amplitude. Nevertheless, NADH (2 mmol/L) significantly diminished spontaneous Ca^{2+} release and Ca^{2+} waves (n = 4 cardiomyocytes), similar to the observations made at room temperature. Preincubation of permeabilized myotubes with rotenone completely negated the inhibitory actions of NADH on transient amplitude and significantly diminished its negative influence on the frequency of spontaneous Ca^{2+} transients (20% versus 32% reduction; Figure 3C).

Confocal line-scan images and F/F₀ plots of Ca^{2+} release induced by application of 20 mmol/L caffeine after introducing NADH (2 mmol/L) were slightly larger (8 ± 4%; n = 12), although not significantly different from caffeine responses of control cells not pretreated with NADH (not shown). Because under these conditions NADH produced a significant decrease of Ca^{2+} wave amplitude and frequency, it is unlikely that the inhibitory actions of NADH were the consequence of a reduced SR Ca^{2+} load. The suppressive actions of NADH on spontaneous Ca^{2+} events seen in permeabilized cardiomyocytes have the same properties as those observed at the level of CICR and single RyR2 channel reconstituted in BLM. These results provide the first direct evidence linking negative regulation of CICR in cardiomyocytes with an NADH oxidase closely associated with the RyR2 complex.

Cardiac SR NADH Oxidase Is Functionally Distinct From Mitochondrial Complex I

The kinetics and specificity of the SR NADH oxidase vis-à-vis mitochondrial oxidases was addressed in several experiments. Cardiac SR preparations demonstrated a [3H]ryanodine-binding site density of 3.61 ± 0.52 pmol/mg protein (Figure 4A), indicating enrichment of junctional SR. Preparations of mitochondrial particles, by contrast, had 14-fold lower [3H]ryanodine-binding site density (0.25 ± 0.06 pmol/mg protein). In addition, the activity of succinate dehydrogenase (SDH), a specific marker of mitochondrial inner membrane, was ≈4-fold higher in preparations of cardiac mitochondrial particles compared with corresponding SR fractions isolated from the same hearts (329 ± 21 versus 88 ± 1.9 μmol/mg protein per minute; Figure 4B). Given a recent report, we cannot discount the possibility that the [3H]ryanodine-binding sites measured in our mitochondrial preparation reflect small amounts of RyR that may be localized within the mitochondrial inner membrane.

To further assess the catalytic properties of SR and mitochondrial particle preparations, oxidation of NADH was measured in the presence and absence of exogenous electron acceptors. Cardiac SR membranes possessed a very high basal rate of oxidation of NADH to NAD⁺+H⁺ (4.97 ± 0.4 μmol/mg protein per minute at 50 μmol/L NADH) in the absence of exogenously introduced electron acceptors (Figure 5A). Moreover, addition to the reaction of either decylubiquinone (electron acceptor for complex I) or cytochrome c (electron acceptor for complex III) failed to enhance the rate of catalysis by cardiac SR. In contrast, cardiac preparations enriched in mitochondrial particles exhibited 6.5-fold lower basal NADH oxidase activity (0.76 ± 0.07 μmol/mg protein per minute) in the absence of exogenously added terminal electron acceptors when assayed at subsaturating substrate concentration (50 μmol/L NADH; Figure 5A).
Sonication of cardiac mitochondrial or SR preparations, a technique commonly used to unmask respiratory activities in intact mitochondria, did not enhance basal NADH oxidase activity in either preparations of cardiac mitochondria or SR, but rather significantly decreased the basal rate of NADH oxidation in a manner dependent on the energy output of the sonicator (Figure 5B). In Figure 5C, the response of NADH oxidase of mitochondria and SR preparations to exogenous decylubiquinone were further examined before and after sonication (40% maximum output). Before sonication, basal mitochondrial NADH oxidase activity increased 2-fold with addition of decylubiquinone, whereas the activity of SR decreased 25%. After sonication, addition of decylubiquinone to cardiac mitochondrial preparations significantly increased the rate of NADH oxidation (nearly 5-fold), whereas the activity of the SR preparations was reduced by about 20% (Figure 5C). These results indicate that the significant higher NADH oxidase activity (approximately 10-fold when measured with 50 μmol/L NADH) found in SR compared with cardiac mitochondrial particles could not be the result of a higher content of NADH-impermeant intact mitochondria in the latter. Moreover, the differential response of the preparations to exogenous decylubiquinone provides additional evidence of the fundamentally different biochemical properties of these NADH oxidases and further supports the absence of intact mitochondria in these SR preparations.

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Further analysis revealed that, unlike SR, mitochondrial NADH oxidase activity was significantly enhanced by addition of not only 15 μmol/L decylubiquinone (from 0.76±0.07 to 1.22±0.13 μmol/mg protein per minute) but also 15 μmol/L cytochrome c (to 2.06±0.16 μmol/mg protein per minute) (Figure 5A). As expected of the NADH-ubiquinone oxidoreductase activity of rat mitochondrial preparations exhibited classic complex I pharmacology in that it was fully inhibited by rotenone (20 nmol/L) but unaffected by antimycin A (50 nmol/L), a complex III inhibitor. Conditions are the same as in A. Data are mean±SE of n=4 determinations each performed in duplicate. Activities are given as μmol NADH/mg protein per minute.

**Figure 5.** NADH oxidase from cardiac SR and mitochondrial particles differ in their substrate requirements. A, In the absence of exogenous electron acceptor decylubiquinone (mitochondrial complex I) or cytochrome c (mitochondrial complex III), the basal NADH oxidase activity of junctional SR membranes (50 μg protein) was nearly 6-fold higher than in mitochondrial particles ([NADH]=50 μmol/L). Both electron acceptors (15 μmol/L) significantly enhanced NADH oxidation in mitochondrial particles but had no effect on SR preparations. Data shown are mean±SE of n=5 determinations each performed in duplicate (*P<0.02, **P<0.0005 vs control). B, After isolation of cardiac mitochondrial particles, the preparations were subjected to sonication at the indicated energy output to further disrupt any remaining intact mitochondria. Basal NADH oxidase activity decreased in a manner dependent on the vigorousness of sonication (40% and 80% of maximum output), indicating disruption of membrane integrity. Data are averages of 3 experiments from 3 preparations each performed in duplicate. C, After sonication (40% of maximal output), the NADH oxidase activity of mitochondrial particles and SR membranes was measured in the presence and absence of exogenously added decylubiquinone (15 μmol/L). Although sonicaton decreased the basal NADH oxidase activity of mitochondrial preparations, addition of exogenous decylubiquinone significantly enhanced NADH-ubiquinone oxidoreductase activity. Data shown are the mean±SD of n=3 independent determinations using preparations different from those reported in A. NADH oxidase activity associated with SR preparations was inhibited by addition of decylubiquinone regardless of sonication. D, NADH-ubiquinone oxidoreductase activity of rat mitochondrial preparations exhibited classic complex I pharmacology in that it was fully inhibited by rotenone (20 nmol/L) but unaffected by antimycin A (50 nmol/L), a complex III inhibitor. Conditions are the same as in A. Data are mean±SE of n=4 determinations each performed in duplicate. Activities are given as μmol NADH/mg protein per minute.
an NADH oxidase activity that copurified with junctional SR enriched with RyR2 that was not dependent on exogenously added electron acceptor and was pharmacologically distinct from the electron transport enzymes characteristic of cardiac mitochondrial particles.

In the absence of exogenously added electron acceptors, the rate of NADH oxidation by cardiac SR was dependent on the concentration of NADH between 50 and 420 μmol/L, and exhibited saturating kinetics with $K_m = 81.5 \pm 14.5$ μmol/L and $V_{max} = 8.8 \pm 0.5$ μmol/mg protein per minute (Figure 6A; mean±SD of n=3 SR preparations). The rapid decrease in optical density at 340 nm was paralleled by an increase in absorbance at 260 nm consistent with concurrent formation of NAD⁺. The NADH analog, deamino-NADH, was oxidized by cardiac SR with the same kinetics as the parent substrate ($K_m = 84.6 \pm 9.4$ μmol/L and $V_{max} = 9.2 \pm 0.6$ μmol/mg protein per minute; mean±SD of n=3 SR preparations). In contrast, NADPH was not a substrate for cardiac SR under the assay conditions used. Unlike cardiac SR, skeletal SR isolated from the same animal oxidized NADH at very slow rate ($V_{max} < 0.5$ μmol/mg protein per minute). Furthermore, in the absence of an exogenous electron acceptor, the cardiac mitochondrial particles exhibited low capacity for NADH oxidation at all substrate concentrations tested ($V_{max} \approx 0.85$ μmol/mg protein per minute; Figure 6A). The activity of NADH-ubiquinone oxidoreductase from mitochondrial particles isolated from the same cardiac tissue was also measured under identical assay conditions and exhibited kinetics consistent with previously published reports with rat cardiac mitochondrial particles ($K_m = 12.9 \pm 1.7$ μmol/L and $V_{max} = 1.6 \pm 0.1$ μmol/mg protein per minute) that were distinct from those of SR.

Pharmacology of the Cardiac SR NADH Oxidase

In comparison with NADH-ubiquinone and cytochrome c oxidoreductases observed with mitochondrial particles (Figures 5A and 5D), the NADH oxidase of cardiac SR showed a distinct pharmacology. Rotenone (the classic blocking agent for NADH-ubiquinone oxidoreductase) inhibited NADH oxidation by cardiac SR with an IC₅₀ ≈ 3.4 ± 0.4 μmol/L (Figure 6B). Pyridaben, another selective inhibitor of this enzyme, was slightly more potent than rotenone itself with an IC₅₀ = 2.2 ± 0.2 μmol/L. NADH oxidation by cardiac SR was also potently inhibited by antimycin A (IC₅₀ = 13.3 ± 0.7 μmol/L), a commonly used inhibitor of NADH-cytochrome c oxidoreductase of mitochondrial complex III (Figure 6B). Finally, diphenyleneiodonium chloride, an inhibitor of flavoenzymes, suppressed NADH oxidation by cardiac SR with IC₅₀ = 27.8 ± 1.6 μmol/L.

The influence of oxygen tension on SR NADH oxidase activity was examined. Purging the sealed reaction mixture with argon (pO₂ ≈ 10 mm Hg) reduced SR NADH oxidase activity to 51 ± 6% of that observed with ambient conditions (pO₂ ≈ 150 mm Hg). Cardiac RyR2 is modulated by nitrosylation. We further explored the influence of the NO donor spermine NONOate on SR NADH oxidase activity. At low pO₂, as little as 5 μmol/L NONOate produced 93% inhibition (IC₅₀ ≈ 0.6 μmol/L), and inhibition was prevented by NO scavenger carboxy-PTIO (not shown). Spermine tetrahydrochloride, which is not a nitrosylating agent, had no effect on catalysis. These findings indicate that NADH oxidase activity may be coordinately regulated with RyR2 by nitrosylation reactions.

PSST-Like Protein of Cardiac SR NADH Oxidase

Is a Molecular Target of Pyridaben

Whether the NADH oxidase identified in the present study was an integral property of the RyR2 homotetramer or a closely associated protein was examined with the photoaffinity probe [3H]TDP. Using electron transport particles isolated from bovine cardiac mitochondria, [3H]TDP was previously shown to selectively bind to a 23-kDa PSST protein, a component of mitochondrial complex I. In the present study, [3H]TDP also selectively labeled a single 23-kDa protein in preparations of cardiac SR and mitochondrial particles (Figure 7, top and middle panels). In spite of the 4-fold lower SDH activity found in SR compared with mitochondrial particles (Figure 4B), SR possessed 23%
higher density of [3H]TDP-binding sites (n = 7). The binding was deemed specific because rotenone (2 μmol/L) completely blocked labeling of the 23-kDa protein by [3H]TDP in all preparations (not shown). [3H]TDP (10 nmol/L) gave 21% specific labeling of skeletal compared with cardiac SR (Figure 7, bottom panel).

NADH-ubiquinone oxidoreductase of mammalian mitochondria possesses a nuclear-encoded PSST subunit that is homologous to NQO6 of bacteria and share a conserved inhibitor-binding site for pyridaben.36,37 The PSST subunit plays a key role in electron transfer by functionally coupling iron-sulfur cluster N2 to quinone. The present results reveal that PSST or a PSST-like protein and NADH oxidase are closely associated with SR preparations enriched in RyR2 complex, but not with RyR1 complex enriched skeletal muscle SR. The exact role of the 23-kDa protein in mediating SR NADH oxidase activity and its relationship to the RyR2 complex remains to be elucidated. Nevertheless, both RyR2 and NADH oxidase (present work) of cardiac SR appear to be under tight regulation by NO. Interestingly, NO inhibits the enzyme, thereby relieving negative modulation by NADH, and enhances RyR2 activity by direct nitrosylation.35 In this regard, NADH oxidase appears to be a more sensitive target of NO.

Significance of Cardiac SR NADH Oxidase
The present findings have identified an NADH oxidase activity that colocalizes with markers of junctional SR isolated from cardiac muscle. There are several fundamental differences between NADH oxidase activity of cardiac SR compared with the classic NADH-ubiquinone and NADH-cytochrome c oxidoreductases of complex I and complex III, respectively, typically measured in isolated electron transport particles of mitochondria. Perhaps the most striking difference observed with the SR enzyme is that catalysis is independent of electron acceptors decylubiquinone or cytochrome c, yet maintains exquisite sensitivity to both complex I and III inhibitors. These inhibitors block NADH oxidation and negate inhibitory modulation by NADH, even at the level of single RyR2 channels reconstituted in BLM. These results indicate that NADH oxidation is not only tightly linked to the RyR2 complex, but necessary for affecting inhibition.

Although the nature of the electron acceptor within SR has not been elucidated in this study, one possible mediator of electron transfer is revealed from photoaffinity labeling studies with [3H]TDP, a PSST-like 23-kDa protein. RyR complexes of skeletal and cardiac muscle have been shown to possess highly reactive sulfhydryl moieties38 that appear to play a redox-sensing function,17,18 and are easily oxidized in vitro.39,41 Consistent with these observations, SR NADH oxidase activity is extremely responsive to pO2 and nitrosylating agent. A reducing environment within the cellular cytosol established by a redox buffer of glutathione where GSH>SSG has been proposed to maintain hyperreactive cysteine moieties in a reduced state and contribute negative modulation of CICR. Redox active quinones (eg, naphthoquinones), by contrast, promote oxidation of hyper-reactive cysteine moieties of RyR1 and RyR2 and enhance channel activity.23,40,41 The present results identify a NADH oxidase functionally associated with the RyR2 complex that participates in electron transfer from NADH to the receptor complex, thereby providing a catalytic mechanism for local negative control of CICR in the heart.

Conclusion
This investigation reveals a negative-feedback control in cardiac muscle that utilizes the very same Ca2+ signals that have been proposed to signal feed-forward control of cellular...
energetic state by regulation of NADH concentration ([NADH]m) within mitochondria in response to work.\textsuperscript{2,10} The [NADH]m of mammalian cells is a key regulatory signal linking rates of oxidative phosphorylation with increasing work loads, especially during conditions of nonlimiting [ADP].\textsuperscript{3–5} Several mechanisms have been proposed whereby changes in mitochondrial Ca\textsuperscript{2+} in turn control [NADH]m. Brandes and Bers\textsuperscript{2} suggest that the regulation of [NADH]m in cardiac muscle involves at least two control mechanisms. When the workload is abruptly increased, [NADH]m initially drops, slowly recovers, and subsequently becomes elevated after the work level returns to the control level. Dynamic changes in cytoplasmic [NADH]/[NAD\textsuperscript{+}] could mirror those of mitochondria and provide an important feedback-control point for SR Ca\textsuperscript{2+} release through the modulation of RyR2 complexes (Figure 8). Finally, increases in cellular [NADH] are observed in several pathophysiological states, including diabetes, heart failure, ischemia, and hypoxia.\textsuperscript{33–46} Thus, [NADH]/[NAD\textsuperscript{+}] ratios may be an important regulatory component that limits SR Ca\textsuperscript{2+} release and contractile function in these conditions.

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