Skeletal muscle sarcoplasmic reticulum contains a NADH-dependent oxidase that generates superoxide

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Xia, Ruohong, Jason A. Webb, Lisa L. M. Gnall, Kerry Cutler, and Jonathan J. Abramson. Skeletal muscle sarcoplasmic reticulum contains a NADH-dependent oxidase that generates superoxide. Am J Physiol Cell Physiol 285: C215–C221, 2003. —Skeletal muscle sarcoplasmic reticulum (SR) is shown to contain an NADH-dependent oxidase (NOX) that reduces molecular oxygen to generate superoxide. Its activity is coupled to an activation of the Ca2+ release mechanism, as evident by stimulation in the rate of high-affinity ryanodine binding. NOX activity, coupled to the production of superoxide, is not derived from the mitochondrion but is SR in origin. The SR preparation also contains a significant NADH oxidase activity, which is not coupled to the production of superoxide and appears to be mitochondrial in origin. This mitochondrial component is preferentially associated with the terminal cisternae region of the SR. Its activity is inhibited by diphenylene iodonium (10 μM), antimycin A (200 nM), and rotenone (40 nM) but is not coupled to the generation of superoxide or the stimulation of the ryanodine receptor. The rate of superoxide production per milligram of protein is larger in SR than in mitochondria. This NOX may be a major source of oxidative stress in muscle.

NADH-dependent superoxide production; ryanodine receptor; oxidative stress; sarcoplasmic reticulum

A MAJOR INTERNAL THREAT to cellular Ca2+ homeostasis of aerobic organisms arises from reactive oxygen species (ROS) and the by-products generated from oxygen metabolism. A number of studies have detailed the effects of one or more ROS on whole muscle tissue or on isolated sarcoplasmic reticulum (SR) from smooth, cardiac, and/or skeletal muscle (12, 30, 33, 34). Skeletal muscle continuously produces superoxide at a low rate, which is dramatically increased with contractile activity (26). Moreover, low ROS levels present under basal conditions are essential for normal force production. Depletion of superoxide and H2O2 by addition of superoxide dismutase (SOD) and catalase, respectively, causes force output to fall (26). Conversely, ROS supplementation causes force output to increase (26). During strenuous exercise, it is believed that high levels of ROS generated are at least partially responsible for muscle fatigue. Superoxide is a by-product of mitochondrial electron transport and the oxidation of xanthine to uric acid catalyzed by xanthine oxidase. It has been shown that O2- at submicromolar levels (generated by addition of xanthine, xanthine oxidase) stimulates the SR Ca2+ release mechanism and displaces calmodulin from cardiac muscle SR (15).

Through well-characterized electron exchange reactions, superoxide gives rise to hydrogen peroxide and subsequently to hydroxyl radicals (in the presence of iron) (37). The cell protects itself from oxidative damage by rapidly converting superoxide to hydrogen peroxide (via the action of SOD) and peroxide into water [utilizing glutathione peroxidase (GPX) or catalase]. Nonenzymatic antioxidants such as reduced glutathione (GSH), vitamins E and C, β-carotene, and α-lipoic acid all protect muscle against oxidative stress.

The SR is a subcellular organelle that controls the contractile state of muscle by regulating the Ca2+ concentration in the cytosol. By hydrolysis of ATP, the SR actively accumulates Ca2+ into its lumen, which leads to muscle relaxation. Depolarization of the transverse tubule membrane results in the release of Ca2+ from the SR and muscle contraction. The Ca2+ release protein is pharmacologically characterized by its ability to bind the plant alkaloid ryanodine with high affinity and high specificity, and, hence, this protein is now known as the ryanodine receptor (RyR). [3H]Ryanodine has been used to identify the Ca2+ release protein and is important in characterizing this receptor (14, 23). It has been repeatedly demonstrated that reagents that open the Ca2+ release channel increase equilibrium binding of ryanodine. The binding of ryanodine has become a functional probe to characterize the open vs. closed state of the Ca2+ release mechanism.

The SR Ca2+ release mechanism has been shown to be a potent target of oxidative modification. It is well established that oxidation of critical thiol groups activates the Ca2+ release mechanism, whereas addition of thiol-reducing agents closes down the Ca2+ channel (2, 20, 35). Oxidative modification of Ca2+ channel function has been observed at the level of skinned fibers, in Ca2+ flux measurements, in single channel measurements, and at the level of high-affinity ryanodine-

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binding measurements (1, 2, 20, 28, 35). It has recently been shown that the RyR is sensitive to the local redox potential (10, 36). More positive redox potentials sensitize the receptor to activation by Ca\(^{2+}\). Under mild oxidative stress, small changes to the cellular redox potential result in significant stimulation of the RyR. Redox reactions may play a critical role in controlling the kinetics of the Ca\(^{2+}\) release mechanism.

It has also been observed that ROS activate Ca\(^{2+}\) release from SR (3, 9, 31) and may act as redox active signaling molecules to activate Ca\(^{2+}\) transport (34). Although it is well known that superoxide is a normal byproduct of mitochondrial electron transport (8), which accounts for ~3% of total O\(_2\) consumption, and that O\(_2^*\) can be generated by xanthine oxidase under ischemic-refusperfusion conditions in cardiac muscle, no previous study has shown that the SR membrane contains an enzymatic mechanism for synthesizing O\(_2^*\). In this article, it is shown that skeletal muscle SR contains an NADH-dependent oxidase that generates O\(_2^*\), which in turn activates the SR Ca\(^{2+}\) release mechanism.

**EXPERIMENTAL PROCEDURES**

SR vesicles were isolated from rabbit fast-twitch skeletal muscle according to the method of MacLennan (19) with small modifications. Dithiothreitol (50 \(\mu\)M) and 0.2 \(\mu\)g/ml leupeptin were included in all buffers except for the final SR resuspension buffer. Samples were stored in liquid N\(_2\).

SR was further fractionated on a discontinuous sucrose gradient (27). The following sucrose solutions (percent by weight) plus 10 mM HEPES, pH 7.0, were layered sequentially in a SW28 centrifuge tube (Beckman): 4 ml of 45%, 7 ml of 40%, 12 ml of 35%, 7 ml of 30%, and 4 ml of 27%. Thirty milligrams of unfractionated SR were layered on top of the gradient and then spun at 22,000 rpm overnight. The heavy fraction (HSR) and light fraction (LSR) were processed as previously described (27) and then stored in liquid N\(_2\).

The Ca\(^{2+}\) release protein, RyR1, was isolated according to the method of Lai et al. (17) with small modifications. Sixty milligrams of SR were suspended in 10 ml of 0.2 M NaCl, 150 \(\mu\)M CaCl\(_2\), 100 \(\mu\)M EGTA, 25 mM PIPES, 1.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 100 \(\mu\)M diithiobisretiol, 2 \(\mu\)g/ml leupeptin, 0.5 mM PMSF, and 3 mg/ml phosphatidylycholine (PC), pH 7.1, and stirred on ice for 2 h. The solubilized SR was then spun at 40,000 rpm for 60 min in a Beckman Ti50 rotor (100,000 \(\times\) g), and ~10 mg of the supernatant were loaded onto 30 ml of a 5–20% linear sucrose gradient containing 0.2 M NaCl, 0.9% CHAPS, 4 mg/ml PC, 150 \(\mu\)M CaCl\(_2\), 100 \(\mu\)M EGTA, 1 \(\mu\)g/ml leupeptin, 0.5 mM PMSF, and 40 mM Tris, pH 7.1. The sucrose gradient was centrifuged at 25,000 rpm (Beckman SW28 rotor) for 18 h at 2°C. Seventeen fractions were pumped off the gradient and assayed for ryanodine binding, NOX activity, and NADH-dependent O\(_2^*\) production. Samples were stored in liquid N\(_2\).

The initial rate of \([^3H]ryanodine binding was determined from time-dependent measurements at 3, 6, 9, and 12 min at \(37°C\). SR at 1.0 mg/ml was incubated at 37°C in binding buffer containing 250 mM KCl, 15 mM NaCl, 50 \(\mu\)M free Ca\(^{2+}\) (buffered with 50 \(\mu\)M EGTA), and 20 mM PIPES, pH 7.1. The time-dependent reaction was initiated by dilution into an equal volume of binding buffer containing 8 nM \([^3H]ryanodine, 50 \(\mu\)M free Ca\(^{2+}\), and various concentrations of NADH, NADPH, NADP, or NAD\(^+\). In all Ca\(^{2+}\)-dependent measurements, Ca\(^{2+}\) was buffered with 50 \(\mu\)M EGTA to a free Ca\(^{2+}\) concentration as calculated by WinMaxx (6). The binding reaction was quenched by rapid filtration through Whatman GF/B filters mounted on a 48-well Brandel cell harvester. Filters were rinsed twice with binding buffer containing 50 \(\mu\)M Ca\(^{2+}\). Scintillation vials were filled with scintillation fluid, shaken overnight, and counted the next day.

**NADH oxidase.** The initial rate of NADH oxidase activity was measured at varying concentrations of NADH by monitoring the absorbance at 338 nm vs. time. The extinction coefficient of 6.25 mM\(^{-1}\) cm\(^{-1}\) was used to convert from absorbance units to concentration of NADH. Experiments were carried out with either rabbit skeletal muscle or rabbit heart mitochondria at a protein concentration of 0.1 mg/ml at room temperature in 50 mM potassium phosphate and 10 mM KCl, pH 7.4 (phosphate buffer). The rate of NADH-dependent oxidase was normalized per milligram of protein.

**Superoxide production.** O\(_2^*\) was measured according to the method of Azzi et al. (4), using the reduction of acetylated ferricytochrome c (AFC) as a measure of O\(_2^*\) production. The initial rate of AFC reduction was determined by monitoring the absorbance difference at 550–540 nm as a function of time and using the extinction coefficient of 16.8 mM\(^{-1}\) cm\(^{-1}\). The reaction was initiated by addition of the indicated concentration of NADH. The assay was carried out at 0.1 mg/ml of either SR or mitochondria with 80 \(\mu\)g/ml AFC (Sigma Chemical) in 50 mM potassium phosphate buffer at room temperature. The rate of production of superoxide was determined by subtracting the rate of reduction of AFC in the presence of SOD (300 U/ml) from the rate in the absence of SOD. Measurements of NADH oxidase activity (338 nm) and cytochrome c reduction (550–540 nm) were simultaneously monitored using an HP 8452A diode array spectrophotometer. A calibration curve was generated in which the absorbance difference between 550 and 540 nm was plotted vs. the concentration of AFC (not shown). The data were then corrected for the low concentration of AFC used in these experiments (80 \(\mu\)g/ml, only 17% of the superoxide produced was detected).

**Measurement of mitochondrial copurification with SR.** Mitochondria were isolated from rabbit heart ventricles (21) and stored in liquid N\(_2\). Polyacrylamide (5%) gels were run according to the method of Laemmli (16). Varying amounts of SR vesicles (10, 20, 40, and 80 \(\mu\)g), and mitochondria (1.25, 2.5, 5.0, and 10 \(\mu\)g) per lane were electrophoresed and stained with either Coomassie blue or transferred onto nitrocellulose paper (Schleicher and Schuell). The nitrocellulose paper was blocked with 5% powdered milk and washed with Tris-buffered saline (TBS). The transfer was then incubated overnight in 0.5 \(\mu\)g/ml anti-F1F0-ATPase mouse IgG (Molecular Probes, Eugene, OR; cat. no. A-21350). The transfer was washed with TBST (TBS with 0.05% Tween 20) and incubated for 30 min in alkaline phosphatase-linked anti-mouse secondary antibody (Sigma-A 3562) diluted 1:20,000. Color was visualized using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as described by the manufacturer (Promega). The transfer was scanned with a Molecular Dynamics Typhoon 9200 imager. The integrated area of the F1F0-ATPase was plotted vs. the amount of added protein per lane. These plots were linear (\(r^2 > 0.97\)), and their slopes were used to estimate the amount of mitochondria in our SR preparation (4.1 ± 1.3%, mean ± SD). This value was obtained from measurements made with three different SR preparations. Similar measurements were repeated with light and heavy SR vesicles to determine the
amount of F₁F₀-ATPase associated with SR derived from the longitudinal and the terminal cisternae region of the SR.

Statistics. All figures show means ± SD. Student’s t-test was used to determine whether the differences in mean values were statistically significant at a P < 0.05 or P < 0.01, as indicated.

RESULTS

Addition of 1.0 mM NADH to SR vesicles resulted in a more than twofold activation of the rate of high-affinity ryanodine binding (Fig. 1). Averaged over all Ca²⁺ concentrations from 1 μM to 1 mM, the degree of activation by NADH over control was 2.17 ± 0.43 (mean ± SD). At the same concentration, NADPH was only half as effective in stimulating the rate of ryanodine binding (degree of activation = 1.63 ± 0.20). All data points at 1 μM Ca²⁺ or greater (with the exception of those treated with NADH + SOD) show statistically significant different mean values compared with controls (P << 0.01). The oxidized forms of the nicotinamide adenine dinucleotides, NAD⁺ and NADP⁺, had no effect on the rate of binding compared with controls (not shown). SOD at 300 U/ml significantly decreased the degree of activation by NADH (degree of activation = 1.14 ± 0.11) but did not decrease the rate of binding back down to control levels. The difference between samples treated with NADH + SOD and control values was statistically significant (P < 0.05) only at Ca²⁺ concentrations between 30 and 100 μM. Further addition of catalase (300 U/ml) had no further effect on the Ca²⁺-dependent rate of ryanodine binding (not shown). H₂O₂ did not appear to be responsible for receptor activation. It appears as if a significant fraction of NADH-dependent activation of ryanodine binding is caused by -O₂⁻. Addition of xanthine oxidase and xanthine resulted in a similar stimulation of Ca²⁺-dependent ryanodine binding. None of these pyridine nucleotides alter the Ca²⁺ dependency of either activation at low micromolar concentrations or inactivation at submillimolar concentrations of Ca²⁺. Neither rotenone (40 nM) nor antimycin A (200 nM) had any effect on NADH (1 mM)-induced activation of ryanodine binding (not shown). NADH-induced activation of ryanodine binding was partially reversed by subsequent treatment with reduced glutathione (GSH) or DTT at concentrations of <1 mM, which indicates that superoxide activates the RyR by oxidizing protein thiols (not shown).

It has previously been shown that the hyperreactive thiols associated with RyR1 have a well-defined redox potential that is sensitive to the open vs. closed state of the channel. At 50 μM, Ca²⁺ the redox potential of the receptor (E_red) is ~−157 mV (36). Addition of channel inhibitors results in a shift of E_red to more positive values, whereas addition of channel activators shifts E_red to more negative values. As shown in Fig. 2, addition of 1 mM NADH shifts the redox potential to

Fig. 1. Ca²⁺-dependent ryanodine binding is stimulated by NADH. Time-dependent ryanodine binding was measured as described in EXPERIMENTAL PROCEDURES. The initial rate of binding (pmol·mg⁻¹·min⁻¹) was calculated from a linear regression fit of binding measurements at 3, 6, 9, and 12 min. Measurements were made in a buffer containing 250 mM KCl, 15 mM NaCl, and 20 mM PIPES, pH 7.1, at a protein concentration of 0.5 mg/ml at 37°C. Measurements were made with either 1 mM NADH (●), 1 mM NADPH (○), 1 mM NADH + 300 U/ml SOD (●), or control (■). This experiment was repeated 10 times on 3 different sarcoplasmic reticulum (SR) preparations with almost identical results. The values shown are means ± SD. The control data points were derived from 6 independent experiments. The NADH and NADH + superoxide dismutase (SOD) data were derived from 4 experiments. The xanthine/xanthine oxidase and the NADPH data were derived from 3 experiments.

Fig. 2. Ryanodine receptor type 1 from skeletal muscle (RyR1) redox potential is shifted to more negative values by NADH. The initial rate of ryanodine binding was measured as described in Fig. 1, at different solution redox potentials (E_red) as previously described (36). All measurements were made at 50 μM Ca⁰⁺ at 37°C with 0.5 mg/ml SR vesicles, either in the presence of 1 mM NADH (●), or in the absence of NADH (■). The values shown are means ± SD derived from 3 experiments.
This shift, induced by \( \cdot \text{O}_2^\cdot \), occurs in spite of the significant amount of GSH present in the buffer that sets the redox potential of the solution. Moreover, consistent with this observation, addition of 50 \( \mu \text{M} \) xanthine and 6.25 nM xanthine oxidase activates ryanodine binding (Fig. 1) and shifts \( E_{\text{red}} \) to \(-175 \text{ mV} \) in the absence of NADH (not shown).

As shown in Fig. 3, NADH-dependent activation is strongly \( \text{O}_2 \) dependent. Addition of SOD decreased the rate of receptor binding. A decrease in oxygen tension was even more effective in inhibiting the binding of ryanodine. Addition of the detergent CHAPS increased binding rates, in the absence of NADH, approximately fourfold. However, the NADH dependency of the rate of ryanodine binding was not altered by CHAPS. The degree of activation by NADH is similar between control and CHAPS-solubilized SR vesicles. As was shown in Fig. 1, the activation by NADH was significantly different from the control. Activation of the receptor was less evident in the presence of SOD and at low \( \text{O}_2 \) tensions (Fig. 3).

NADH oxidase activity and the rate of production of \( \cdot \text{O}_2^\cdot \) were measured as described in EXPERIMENTAL PROCEDURES with both SR and mitochondrial preparations. As seen in Fig. 4, 72% of the SR NOX activity is inhibited by 200 nM antimycin A, whereas 94% of the mitochondrial NOX activity is inhibited by the same concentration of antimycin A (Fig. 5). Antimycin A and rotenone are potent inhibitors of electron transport in mitochondria. The \( K_i \) for antimycin A inhibition of SR NADH oxidase activity = 12.5 nM (Fig. 4). The rate of \( \cdot \text{O}_2^\cdot \) production by the SR (3.3 \pm 0.6 nmol\cdot mg\(^{-1}\)\cdot min\(^{-1}\)) was not affected by antimycin A.

Fig. 3. RyR1 activation by NADH is \( \text{O}_2 \) dependent. The initial rate of ryanodine binding was measured as a function of NADH concentrations, as described in Fig. 1. All measurements were made at 50 \( \mu \text{M} \) \( \text{Ca}^{2+} \) at 37°C with 0.5 mg/ml SR vesicles. Control measurements were carried out at ambient \( \text{O}_2 \) concentrations (~21% \( \text{O}_2 \)), in the presence of 1 mg CHAPS/mg SR (○), or 300 U/ml SOD (△). Measurements were also made at reduced \( \text{O}_2 \) tension of 0.5% (×) or at 0.1% (◆). The values shown are means \pm SD and were repeated 3 times, except for the control data, which were repeated 5 times.

Fig. 4. SR NADH oxidase is inhibited by antimycin A. The initial rates of NADH oxidation (●) were determined as a function of antimycin A concentration. All experiments were carried out in 50 mM KH\(_2\)PO\(_4\), pH 7.4, at room temperature at an SR concentration of 0.1 mg/ml. The reaction was initiated by addition of 50 \( \mu \text{M} \) NADH. The values shown are means \pm SD for at least 3 measurements at each antimycin A concentration. NOX, NADH-dependent oxidase.

Fig. 5. Oxidation of NADH by SR and mitochondria. The initial rate of NADH oxidase activity was measured by adding 50 \( \mu \text{M} \) NADH and monitoring the absorbance at 338 nm as a function of time for 200 s in a buffer containing 50 mM KH\(_2\)PO\(_4\), pH 7.4, at room temperature. The assay was carried out with either unfractionated SR (hatched bars), SR derived from the heavy fraction (shaded bars), light fraction (open bars), or mitochondria (solid bars) at a protein concentration of 0.1 mg/ml. Measurements were made in the absence (control) or presence of 200 nM antimycin A (+ A.A). "Inhibited by A.A" is the difference between the control and samples treated with antimycin A. The values shown are means \pm SD of 4 trials. This experiment was repeated on at least 4 different SR preparations with similar results.
level as did rotenone and antimycin A at concentrations of DPI >10 μM. DPI also had no effect on the rate of production of \(-O_2^\cdot\) (not shown).

These observations suggest that a large fraction of the NADH oxidase activity is caused by mitochondria that copurify with the SR preparation, but the generation of superoxide is not caused by the mitochondria. This was further examined by isolating rabbit heart mitochondria (21). Because the SR vesicles used in all studies described in this paper were stored in liquid N\(_2\), the isolated mitochondria were also rapidly frozen in liquid N\(_2\). Freezing and thawing of pigeon heart mitochondria has been shown to dramatically increase NADH oxidase activity by increasing the permeability of the mitochondria to NADH (24). As shown in Fig. 5, the high NOX activity of rabbit heart mitochondria is inhibited by 200 nM antimycin A to 6% of control, whereas a negligible amount of \(-O_2^\cdot\) was produced by the mitochondria (1.3 ± 1.8 nmol·mg\(^{-1}\)·min\(^{-1}\)). SOD had no effect on the NOX activity of the mitochondria.

If the antimycin A inhibitable component of SR NOX activity is caused by mitochondria that copurify with the SR, this should be evident by measuring the amount of mitochondrial proteins in our SR preparation. By assaying for the presence of the F\(_1\)F\(_0\)-ATPase from the inner mitochondrial membrane (see EXPERIMENTAL PROCEDURES), it was determined that 4.1 ± 1.3% of the unfractionated SR that was used in most of the experiments described (Figs. 1–4) is mitochondrial in origin. Given the large NOX activity of the mitochondria (Fig. 5), mitochondria associated with the SR accounts for a significant contribution to the NOX activity of the isolated SR. This is also reflected in the difference in NOX activity of HSR and LSR. As shown in Fig. 5, the ratio of antimycin A inhibitable NOX activity in HSR compared with that in LSR = 2.5 ± 0.6, whereas the ratio of F\(_1\)F\(_0\)-ATPase in HSR compared with LSR = 2.8 ± 1.2. This latter result was obtained by comparing Western blots of HSR and LSR vesicles stained with an antibody to the F\(_1\)F\(_0\)-ATPase (repeated three times). There is a good correlation between the antimycin A-inhibitable component of NOX activity of the HSR vesicles and the increased presence of mitochondria. These observations further support the hypothesis that the antimycin A inhibitable component of NOX activity is caused by mitochondria associated with the SR. This result also suggests that the approximate threefold enhancement of mitochondria in the HSR is a natural consequence of its native association with the terminal cisternae of the SR.

NOX activity associated with the SR that is not inhibited by antimycin A (5.7 ± 0.1 nmol·mg\(^{-1}\)·min\(^{-1}\)) is coupled to the production of \(-O_2^\cdot\) (3.3 ± 0.6 nmol·mg\(^{-1}\)·min\(^{-1}\)). NADH plus AFC in the absence of SR showed a negligible, time-dependent reduction of AFC and no oxidation of NADH. Moreover, SR in the absence of NADH does not reduce cystochrome c; \(-O_2^\cdot\) was not produced in the absence of SR or NADH.

The rate of reduction of AFC has been used to monitor the reduction of \(-O_2^\cdot\) in biological membranes (4). AFC can be reduced by low molecular weight reducing agents other than superoxide (i.e., GSH and semiquinones). However, by measuring the difference in the rate of AFC reduction, in the absence and presence of SOD (300 U/ml), the rate of \(-O_2^\cdot\) production was reproducibly determined.

AFC measures \(-O_2^\cdot\) production on the outside of the SR vesicle. Control experiments were carried out at 1 mg CHAPS/mg SR to solubilize the SR vesicles. The rate of reduction of AFC in the presence of CHAPS was no different than that of experiments carried out in the absence of CHAPS. This indicates that superoxide is exclusively produced on the cytoplasmic face of the SR.

The rate of \(-O_2^\cdot\) production per milligram of SR is ~3.3 ± 0.6 nmol·mg\(^{-1}\)·min\(^{-1}\). This is six to seven times higher than the rate of \(-O_2^\cdot\) production previously measured in the presence of 2 μM antimycin A and 3 mM succinate in submitochondrial particles derived from rat heart measured at the same pH (7). Moreover, unlike mitochondria, the SR preparation showed no measurable \(-O_2^\cdot\) production in the presence of 10 mM succinate, in the absence of NADH (not shown).

To identify which protein or proteins were responsible for the production of superoxide and the oxidation of NADH, a 5–20% sucrose gradient was prepared as described in EXPERIMENTAL PROCEDURES, and various fractions were eluted from the gradient. The highest density fractions were eluted first (fraction 1). As shown in Fig. 6, high-affinity [\(^{3}H\)]ryanodine binding was localized in fractions 2–5. As observed in Fig. 1, with isolated SR vesicles, addition of 1 mM NADH to those fractions containing the RyR resulted in a twofold stimulation in receptor binding. Moreover, NADH oxidase activity also copurifies with those fractions rich in RyR1 (not shown).

![Fig. 6. Activation of the purified RyR1 by NADH. Fractions were eluted from a 5–20% sucrose gradient of CHAPS-solubilized SR as previously described (17). The heaviest fractions were eluted first (lowest fraction numbers). Fifty microliters of each fraction were denatured in standard ryanodine-binding buffer (see Fig. 1) containing 4 mM [\(^{3}H\)]ryanodine for 18 h at 25°C, either in the absence (○) or presence (•) of 1 mM NADH. These experiments were repeated on at least 6 sucrose gradients with very similar results. The data shown are means ± SD.](image-url)
DISCUSSION

The role of ROS in the skeletal muscle contractile process has recently received a great deal of attention. It has been demonstrated that skeletal muscle produces low levels of superoxide during the normal contractile process. As muscle activity increases, so does the generation of superoxide. Depletion of \( \text{O}_2^- \) by addition of superoxide dismutase, or addition of catalase, which results in removal of \( \text{H}_2\text{O}_2 \), causes a decrease in muscle contractility (25, 26). Reintroduction of ROS causes force to increase. Moreover, it has been shown that \( \text{O}_2^- \) at low levels stimulates release of \( \text{Ca}^{2+} \) from the SR membrane, whereas \( \text{O}_2^- \) at higher concentrations inhibits the \( \text{Ca}^{2+} \) release process (15, 25). This inactivation of actin at higher \( \text{O}_2^- \) may be at least partially responsible for muscle fatigue. It has been proposed that an increased level of \( \text{O}_2^- \) measured in the extracellular space during muscle activity might be generated by either the mitochondrion or by xanthine oxidase (32). However, it is unlikely that \( \text{O}_2^- \) generated within the mitochondria could diffuse into the extracellular space or that it would diffuse into the cytosolic space to interact with the SR. Moreover, the xanthine oxidase inhibitor oxypurinol failed to reduce fatigue development and lipid peroxidation in diaphragm muscle (32).

In this article, we show for the first time that skeletal muscle SR contains an NADH-dependent oxidase that produces \( \text{O}_2^- \), which in turn stimulates the SR RyR1. This effect is inhibited by SOD and decreased \( \text{O}_2 \) tension. Moreover, the \( \text{O}_2^- \) produced is not due to mitochondrial contamination. Unlike the mitochondrion, SR’s production of \( \text{O}_2^- \) is not activated by succinate, and it is not affected by either rotenone, antimycin A, or DPI. It is important to recognize that the rate of \( \text{O}_2^- \) production per milligram of protein in SR is higher than the corresponding rate measured in sub-mitochondrial particles derived from rat heart muscle (7).

The EC_{50} for NADH-dependent activation of NOX activity is \( \sim 40 \mu\text{M} \), whereas the EC_{50} for reduction of AFC is \( <2 \mu\text{M} \) (not shown). This large difference in sensitivity to NADH is due to the presence of at least two NADH-dependent oxidases. The one that comprises \( \sim 75\% \) of the oxidase activity has a lower affinity for NADH and is inhibited by antimycin A, rotenone, and DPI. Given the high NOX activity of mitochondria after freezing and thawing and that \( \sim 41\% \) of this SR preparation is mitochondrial in origin, it appears that the majority of the SR NOX activity originates from the associated mitochondria. The remaining NOX activity (5.7 \( \pm \) 0.1 nmole \( \cdot \) mg\(^{-1}\) \cdot min\(^{-1}\)) is coupled to the production of superoxide (3.3 \( \pm \) 0.6 nmole \( \cdot \) mg\(^{-1}\) \cdot min\(^{-1}\)).

The interaction between the SR/endoplasmic reticulum and the adjoining mitochondria has recently received a great deal of attention (11). \( \text{Ca}^{2+} \) spikes associated with \( \text{Ca}^{2+} \) release from the SR are coupled to an increase in the internal mitochondrial \( \text{Ca}^{2+} \) concentration (22). It has also been shown that \( \sim 90\% \) of the \( \text{Ca}^{2+} \) release units in cardiac ventricular myocytes are in close proximity to adjoining mitochondria (29). The observation in this article that there is three times as much mitochondria associated with the HSR than with the LSR suggests that these mitochondria may be functionally associated with the SR and that they represent more than just a contamination resulting from the SR preparation.

The NADH concentration, at which the initial rate of either NADH oxidation or \( \text{O}_2^- \) production is half maximal, is in the low micromolar concentration range (not shown). The concentration of NADH in resting human quadriceps femoris muscle is \( \sim 80 \mu\text{M} \). This level increases approximately threefold after a brief isometric contraction protocol (13). The concentration of NADH in muscle is at least one order of magnitude higher than the \( K_m \) associated with the generation of \( \text{O}_2^- \). The oxidation rate of NADH and the production rate of \( \text{O}_2^- \) appears to be maximal and is therefore not controlled by changes in the cellular NADH concentration. Whether or not the activity of this protein in muscle is controlled by changes in \( \text{O}_2 \) tension or other endogenous modulators is not yet known.

The observation that NADH oxidase activity, ryanodine binding, and activation of ryanodine-binding activity induced by NADH copurifies in sucrose gradient fractions suggests that the RyR1 contains NADH oxidase activity. This hypothesis is supported by a recent publication showing that NAD binds to residues 41–420 of RyR1 with an apparent dissociation constant, \( K_d = 10 \mu\text{M} \) (5). Alternatively, it is possible that a small amount of contaminating protein that copurifies with RyR1 is responsible for NADH oxidase activity and the production of \( \text{O}_2^- \) observed.

Superoxide generated by an endogenous NADH oxidase present in SR increases RyR activity. It has previously been demonstrated that in a similar manner, an increase in the local redox potential activates the RyR (36) and single channel activity of the reconstituted RyR1 (10). A more oxidizing environment sensitizes the \( \text{Ca}^{2+} \) release mechanism to activation. The purpose of having an NADH oxidase capable of generating \( \text{O}_2^- \) may well be to introduce a low level of oxidative stress, which turns up the gain and increases the open probability of the \( \text{Ca}^{2+} \) release channel. Under more severe oxidizing conditions, during fatigue, high levels of ROS may close down the \( \text{Ca}^{2+} \) release mechanism to prevent further rundown of the muscle.

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