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Characteristics and actions of NAD(P)H oxidase on the sarcoplasmic reticulum of coronary artery smooth muscle

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Yi, Xiu-Yu, Victoria X. Li, Fan Zhang, Fan Yi, Daniel R. Matson, Ming Tao Jiang, and Pin-Lan Li. Characteristics and actions of NAD(P)H oxidase on the sarcoplasmic reticulum of coronary artery smooth muscle. *Am J Physiol Heart Circ Physiol* 290: H1136–H1144, 2006. First published October 14, 2005; doi:10.1152/ajpheart.00296.2005.—It has been reported that nonmitochondrial NAD(P)H oxidases make an important contribution to intracellular O_2^- in vascular tissues and, thereby, the regulation of vascular function. Topological analyses have suggested that a well-known membrane-associated NAD(P)H oxidase may not release O_2^- into the cytosol. It is imperative to clarify the source of intracellular O_2^- associated with this enzyme and its physiological significance in vascular cells. The present study hypothesized that an NAD(P)H oxidase on the sarcoplasmic reticulum (SR) in coronary artery smooth muscle (CASM) regulates SR ryanodine receptor (RyR) activity by producing O_2^- locally. Western blot analysis was used to detect NAD(P)H oxidase subunits in purified SR from CASM. Fluorescent spectrometric analysis demonstrated that incubation of SR with NADH time dependently produced O_2^- , which could be substantially blocked by the specific NAD(P)H oxidase inhibitors diphenylene iodonium and apocynin and by SOD or its mimetic tiron. This SR NAD(P)H oxidase activity was also confirmed by HPLC analysis of conversion of NADH to NAD^+ . In experiments of lipid bilayer channel reconstitution, addition of NADH to the *cis* solution significantly increased the activity of RyR/ Ca^{2+} release channels from these SR preparations from CASM, with a maximal increase in channel open probability from 0.0044 ± 0.0005 to 0.0213 ± 0.0018 ; this effect of NADH was markedly blocked in the presence of SOD or tiron or the NAD(P)H oxidase inhibitors diphenylene iodonium, *N*-vanillylnonanamide, and apocynin. These results suggest that a local NAD(P)H oxidase system on SR from CASM regulates RyR/ Ca^{2+} channel activity and Ca^{2+} release from SR by producing O_2^- .

calcium mobilization; vascular myocytes; free radicals

ALTHOUGH THE PRODUCTION OF O_2^- can be induced by activation of NAD(P)H oxidase, xanthine/xanthine oxidase (X/XO), mitochondrial NAD(P)H oxidase, or uncoupled nitric oxide (NO) synthase, there is increasing evidence that nonmitochondrial NAD(P)H oxidases represent a major source of O_2^- in the vessel wall for the redox regulation of vascular endothelial and smooth muscle function (13, 34, 38, 43, 59). It has been estimated that this nonmitochondrial NAD(P)H oxidase-derived O_2^- constitutes >95% of O_2^- production in the vasculature, especially on stimulation (34, 38). NADH and NADPH were used as electron donors to show that this enzyme cata-

lyzes the reduction of two electrons with O_2 to form O_2^- (1, 16, 23). In vascular smooth muscle cells (VSMCs), the nonmitochondrial NAD(P)H oxidase has been demonstrated to share some, but not all, of the characteristics of phagocyte NAD(P)H oxidase, an enzyme that is associated with the plasma membrane and composed of at least five subunits. These subunits include gp91^{phox} and p22^{phox}, two transmembrane proteins that form a stable enzyme complex referred to as cytochrome *b*₅₅₈, and p47^{phox}, p67^{phox}, and p40^{phox}, three subunits in the cytosol in resting cells that are translocated to the cell membrane and activate cytochrome *b*₅₅₈ to function as an oxidase on stimulation. In addition to these subunits, the GTPases Rac2 (Rac1 in some cells) and Rap1A may also participate in the assembly of the active NAD(P)H oxidase complex.

Molecular biological approaches have been used to detect different subunits, such as gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox}, in VSMCs; however, the occurrence of different subunits is dependent on vessel size and cell types. In addition, several different homologs of gp91^{phox}, such as Nox1, Nox4, and Nox5, have been demonstrated in VSMCs. The expression of multiple gp91^{phox} isoforms in VSMCs may determine a compartmental distribution of NAD(P)H oxidase activity and substrate preference. Functionally, many studies demonstrated production of NAD(P)H oxidase in the vasculature or VSMCs, resulting in accumulation of O_2^- within cells when it is activated (12, 23). This intracellular accumulation of O_2^- led to an assumption that a plasma membrane-bound NAD(P)H oxidase may produce and release O_2^- within cells, which is different from the orientation of phagocyte NAD(P)H oxidase (13). However, topological analysis of the Nox subunits indicates that the membrane-associated NAD(P)H oxidase should not release O_2^- into the cytosol (23). Recent studies on subcellular localization of vascular NAD(P)H oxidase also demonstrated that O_2^- within vascular cells may not be derived from plasma membrane NAD(P)H oxidase but, rather, from intracellular compartmental NAD(P)H oxidase (23). To provide direct evidence supporting this view, the present study was designed to determine whether an NAD(P)H oxidase is present on the sarcoplasmic reticulum (SR) of VSMCs and whether this oxidase participates in the regulation of SR function related to intracellular Ca^{2+} release. Because there is considerable evidence that the SR ryanodine receptors (RyRs) may serve as a redox sensor to regulate Ca^{2+} signaling (10, 18,

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48, 54), an NAD(P)H oxidase localized on the SR could play an essential role in the redox regulation of SR function.

We first determined the presence of this nonmitochondrial NAD(P)H oxidase in purified SR from coronary artery smooth muscle (CASM) by Western blot analysis of its different subunits. The enzyme activity in these purified SR fractions was detected by fluorescent spectrometric measurement of NADH-derived O_2^- and HPLC analysis of the conversion of NADH to NAD^+ . Then we determined whether this SR NAD(P)H oxidase-derived O_2^- alters the activity of reconstituted RyR/ Ca^{2+} channels from the SR of CASM. The results from these experiments strongly support the view that SR NAD(P)H oxidase occurs in coronary arterial myocytes, which primarily use NADH as a substrate to produce O_2^- and, thereby, control the activity of the RyR/ Ca^{2+} release channels on the SR in these cells.

MATERIALS AND METHODS

Preparation of purified SR from bovine CASM. Coronary arteries were dissected from the bovine heart, and SR-enriched microsomes (SR membrane) of these arteries were prepared as we described previously (27, 29, 45). Briefly, the dissected coronary arteries (500–1,000 μ m OD) were cleared of surrounding fat and connective tissues. The arteries were cut open along the longitudinal axis and pinned lumen side up to a Sylgard-coated dish containing ice-cold HEPES-buffered physiological saline solution (in mM: 140 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.17 $MgSO_4$, 1.18 NaH_2PO_4 , 5.5 glucose, and 10 HEPES, pH 7.4). A sharp blade was used to scratch endothelial cells from the arteries. Then the segments of the arteries were cut into small (2- to 3-mm-long) pieces and homogenized with a Polytron (Brinkman) in ice-cold MOPS buffer [0.9% NaCl, 10 mM MOPS (pH 7.0), 2 μ M leupeptin, and 0.8 μ M benzamide]. The homogenate was centrifuged at 4,000 g for 20 min at 4°C, and the supernatant was further centrifuged at 8,000 g for 20 min at 4°C and then at 40,000 g for 30 min. The pellet, termed the crude SR membrane, was resuspended in the SR solution (0.9% NaCl, 0.3 M sucrose, and 0.1 μ M phenylmethylsulfonyl fluoride) (27). The crude SR was further fractionated on a discontinuous sucrose gradient (40, 52). The following sucrose solutions (percent by weight) plus 10 mM HEPES, pH 7.0, were layered sequentially in a centrifuge tube (model SW28, Beckman) as follows: 4 ml of 45%, 7 ml of 40%, 12 ml of 35%, 7 ml of 30%, and 4 ml of 27%. Crude SR (30 mg) was layered on top of the gradient, and the tube was spun at 64,000 g overnight. A fraction from 37–40% sucrose contained the purified SR, which was collected and diluted in MOPS solution and subjected to further centrifugation at 40,000 g for 90 min at 4°C. The pellet was resuspended in the SR solution, aliquoted, frozen in liquid N_2 , and stored at $-80^\circ C$.

Western blot analysis of NAD(P)H oxidase subunits. The expression of gp91^{phox}, Nox4 (an isoform of gp91^{phox}), Nox1, p67^{phox}, and p47^{phox} was examined in the crude and purified SR by Western blot analysis with monoclonal anti-gp91^{phox}, anti-p67^{phox}, and anti-p47^{phox} antibodies (Transduction Laboratories) or anti-Nox4 and anti-Nox1 (Santa Cruz). To demonstrate the purity of SR, the binding on the membrane was reprobed with a primary antibody against plasma membrane protein, caveolin, after the membrane was stripped by incubation at 50°C for 30 min in a buffer containing 67.5 mM Tris·HCl (pH 6.8), 100 mM β -mercaptoethanol, and 2% SDS. After they were washed, all membranes were incubated in 5% nonfat dry milk in Tris-buffered saline-Tween 20 and subsequently with a monoclonal antibody against caveolin (1:1,000 dilution for 2 h; Upstate) and then with an anti-mouse IgG as secondary antibody. All washing, probing, and reprobing processes with different antibodies were performed as we described previously (53, 57). The immunoreactive bands of various proteins were visualized by a reaction with detection solution according to the manufacturer's instruction. The

films with immunoreactive blots were scanned by a densitometer, and the intensity of corresponding protein bands was quantitated using UN-SCAN-IT software (Silk Scientific).

Fluorescent spectrometric assay of O_2^- production from SR NAD(P)H oxidase. A modification of methods described previously (33) was used for fluorescence spectrometry of O_2^- production in the SR from coronary arteries. Briefly, the fluorogenic oxidation of dihydroethidium (DHE) to ethidium was used as a measure of O_2^- . The purified SR (20 μ g) prepared from bovine CASM was incubated with 10 μ M DHE and salmon testes DNA (0.5 mg/ml) with or without 1 mM NADH in a microtiter plate at 37°C for 30 min, and then ethidium-DNA fluorescence was measured at 475-nm excitation and 610-nm emission with a fluorescence microplate reader (model FL600, Bio-Tek). The NAD(P)H oxidase activity required to produce O_2^- in the SR was examined by addition of 1 mM NADH as a substrate in the reaction mixture. Salmon DNA was added to bind to ethidium and, consequently, stabilize ethidium fluorescence, thereby increasing the sensitivity of O_2^- measurement (>40-fold). The enzyme activity of NAD(P)H oxidase and O_2^- levels are presented as percent increases in ethidium fluorescence vs. control. These DHE fluorescent spectrometric assays of basal O_2^- levels and NADH oxidase activity were described in our previous studies (26, 56). NADH, rather than NADPH, was used in the present study, because Nox4-containing NAD(P)H oxidase has been reported to preferentially use NADH as substrate (21, 23).

HPLC analysis of NAD(P)H oxidase activity. The activity of NAD(P)H oxidase was also determined by HPLC analysis of the conversion of NADH to NAD^+ , as described previously (30, 39, 47). Purified SR (50–100 μ g) from coronary arteries was incubated with 1 mM NADH at 37°C for 20 min in the absence or presence of the NAD(P)H oxidase inhibitors diphenylene iodonium (DPI, 50 μ M) and apocynin (Apo, 100 μ M) or the superoxide scavengers SOD (200 U/ml) and tempol (0.1 mM). To prevent the further conversion of NAD^+ to cADP-ribose, nicotinamide (5 mM), an ADP-ribosyl cyclase inhibitor, was coincubated in the reaction. After incubation, the reaction mixture was rapidly frozen in liquid N_2 to terminate the reaction. Before analysis with HPLC, the reaction mixtures were ultrafiltered using an Amicon filter to remove the protein.

The HPLC system included a solvent delivery system (model 1090L, Hewlett-Packard, Avondale, PA) and a photodiode array detector (model 1040A, Hewlett-Packard) with a 20- μ l flow cell. Nucleotides were separated on a Supelcosil LC-18T column (3 μ m, 4.6 \times 150 mm) with a Supelcosil LC-18 guard column (5 μ m, 4.6 \times 20 mm; Supelco, Bellefonte, PA). Data were collected and analyzed with a Hewlett-Packard Chemstation as described previously (28). The ratio of NAD^+ to NADH was calculated to represent NAD(P)H oxidase activity.

Reconstitution of SR RyR/ Ca^{2+} release channels into lipid bilayer and recording of channel activity. The purified SR membranes from CASM enriched in RyR/ Ca^{2+} release channels were reconstituted into planar lipid bilayers as described previously (27, 45). Briefly, phosphatidylethanolamine and phosphatidylserine (1:1) were dissolved in decane (25 mg/ml) and used to form a planar lipid bilayer in a 250- μ m aperture between two chambers filled with *cis* and *trans* solutions. The SR membranes (50–100 μ g) were added to the *cis* solution and fused into the lipid bilayer to reconstitute RyR/ Ca^{2+} release channels. The activity of these channels was detected in a symmetrical cesium methanesulfonate solution (300 mM) in all experiments. To increase the channel activity, 1 μ M free Ca^{2+} in the *cis* solution was adjusted by addition of Ca^{2+} standard solution containing $CaCl_2$ and EGTA as described previously (29, 45).

An integrating bilayer clamp amplifier (model BC-525C, Warner Instrument) was used to record single-channel currents in the bilayer. Data acquisition and analysis were performed with pCLAMP software (version 8, Axon Instruments). The channel open probability (NP_o) in the lipid bilayer was determined from 3- to 5-min recordings as

described previously in our patch-clamp studies (27, 28, 45). All lipid bilayer experiments were performed at room temperature ($\sim 20^\circ\text{C}$).

The effect of 0.1–0.5 mM NADH on RyR/ Ca^{2+} release channels of the SR was determined in the absence or presence of SOD (200 U/ml) or tiron (1 mM), a chemical mimetic of SOD that is capable of removing O_2^- from the intracellular and extracellular environment. The effect of 0.1–0.5 mM NADH on RyR/ Ca^{2+} release channel activity was measured in the absence or presence of the NAD(P)H oxidase inhibitors *N*-vanillylonanamide (NVN, 10 μM), Apo (100 μM), and DPI (50 μM) or vehicle. All the compounds used in these experiments were added to the *cis* solution, and currents were recorded at a holding potential of -40 mV.

To determine the site at which O_2^- targets RyR/ Ca^{2+} release channels in the reconstituted bilayer membrane, X/XO, an exogenous O_2^- -producing system, or NADH was added to the *cis* or *trans* solution, respectively. The channel activity was recorded and analyzed as described above.

Statistics. Values are means \pm SE; the significance of the differences in mean values between and within multiple groups was examined using an analysis of variance for repeated measures followed by Duncan's multiple range test. Student's *t*-test was used to evaluate statistical significance of differences between two paired observations. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of NAD(P)H oxidase subunits in the purified SR by Western blot analysis. To confirm the presence of NAD(P)H oxidase on the SR of bovine CASM, Western blot analysis was performed on crude and purified SR prepared from bovine CASM. Arterial homogenates were used as positive control, because most Nox isoforms could be detected in this vascular tissue with different cell types. Four different subunits of NAD(P)H oxidase, Nox4, Nox1, p47^{phox}, and p67^{phox}, were detected with anti-Nox4, anti-Nox1, anti-p47^{phox}, and anti-p67^{phox} antibodies in crude and purified SR from CASM ($n = 4$; Fig. 1). These antibodies recognized the major bands at 65 kDa for Nox4, 65 kDa for Nox1, 47 kDa for p47^{phox}, and 67 kDa for p67^{phox}. No gp91^{phox} was detected in crude and purified CASM SR preparations, but this isoform of Nox was found in arterial homogenates endothelium-intact coronary arteries. When the membrane was probed with an antibody against caveolin, a 24-kDa band was detected in the crude, but not the purified, SR ($n = 4$). This finding confirmed

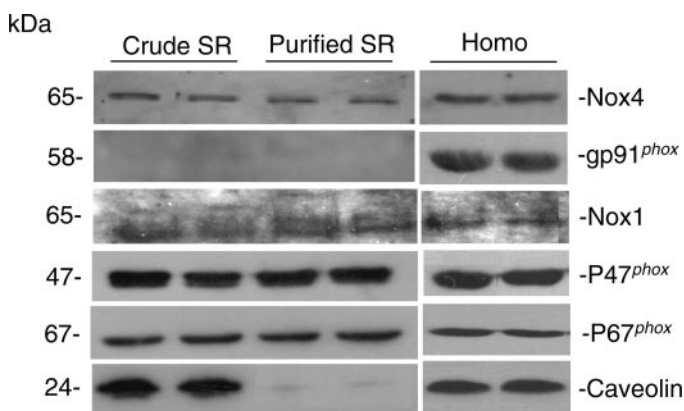


Fig. 1. Western blot of protein expression of NAD(P)H oxidase subunits in crude sarcoplasmic reticulum (SR), purified SR, and homogenate (Homo) of bovine coronary artery smooth muscle (CASM). Caveolin was used to determine purity of SR prepared by gradient centrifugation.

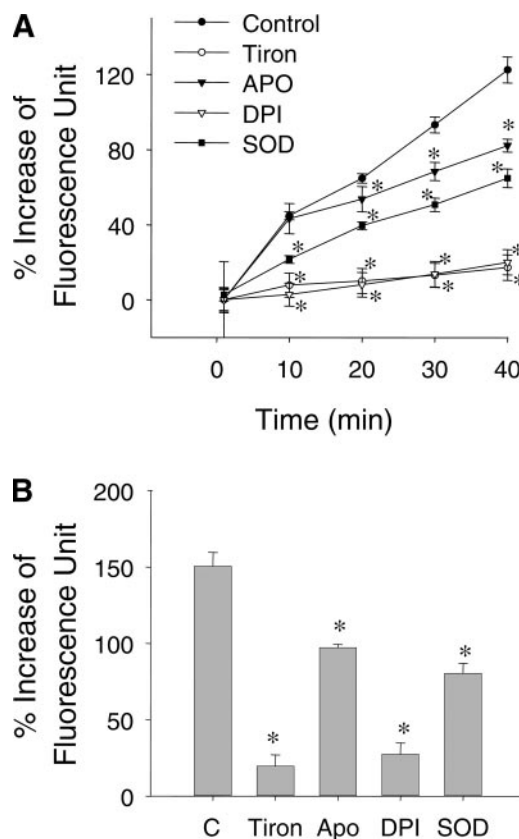


Fig. 2. Time-dependent O_2^- production by purified SR from bovine CASM. **A:** time course of changes in fluorescence intensity of ethidium-DNA complex from oxidation of dihydroethidium by O_2^- in the reaction mixture of the SR with NADH. **B:** maximal O_2^- production under control condition and in the presence of SOD or its mimetic tiron or the NAD(P)H oxidase inhibitors apocynin (Apo) and diphenylene iodonium (DPI). * $P < 0.05$ compared with control (C).

that the purified SR was not contaminated by plasma membrane fractions.

Production of O_2^- via NAD(P)H oxidase measured by fluorescent spectrometry in the SR. To demonstrate that NAD(P)H oxidase is functioning in the purified SR from bovine CASM, NAD(P)H-dependent O_2^- production was measured using NADH as substrate. As discussed above, NADH was chosen as the substrate because of the preference of Nox4-containing NAD(P)H oxidase for NADH, rather than NADPH. Incubation of the SR with NADH time dependently increased O_2^- production (Fig. 2A), which was detected by a time-dependent percent increase in ethidium-DNA fluorescence intensity ($n = 6$). This time-dependent increase of O_2^- production was markedly inhibited by the NAD(P)H oxidase inhibitors Apo (100 μM) and DPI (50 μM) and SOD (200 U/ml) and its mimetic tiron (1 mM) ($n = 6$). The maximal percentage of fluorescence increase corresponding to O_2^- production in this SR preparation is summarized in Fig. 2B. O_2^- -specific fluorescence intensity was dramatically increased in the presence of NADH compared with the SR incubated without NADH (control vs. blank). SOD or its chemical mimetic tiron and the NAD(P)H oxidase inhibitors DPI and Apo substantially attenuated NADH-derived O_2^- production in this SR preparation. The blank response, $\sim 2\%$ increase in fluorescence intensity, was obtained from the SR without

addition of NADH in the reaction mixture, which represents the basal O_2^- production possibly derived from endogenous substrates or other enzymatic pathways (data not shown).

HPLC analysis of NADH conversion to NAD^+ by NAD(P)H oxidase in the SR. Reverse-phase HPLC chromatograms in Fig. 3A depict the production of NAD^+ by NAD(P)H oxidase in the purified SR of bovine CASM. When the SR was incubated with NADH, a product with retention time of 4.3 min coeluted with synthetic NAD^+ (control). In the presence of DPI, the formation of NAD^+ was markedly reduced. The ratio of NAD^+ to NADH in the control group was 0.370 ± 0.065 (Fig. 3B). In the presence of DPI or Apo, the production of NAD^+ was significantly decreased by 86% and 85%, respectively.

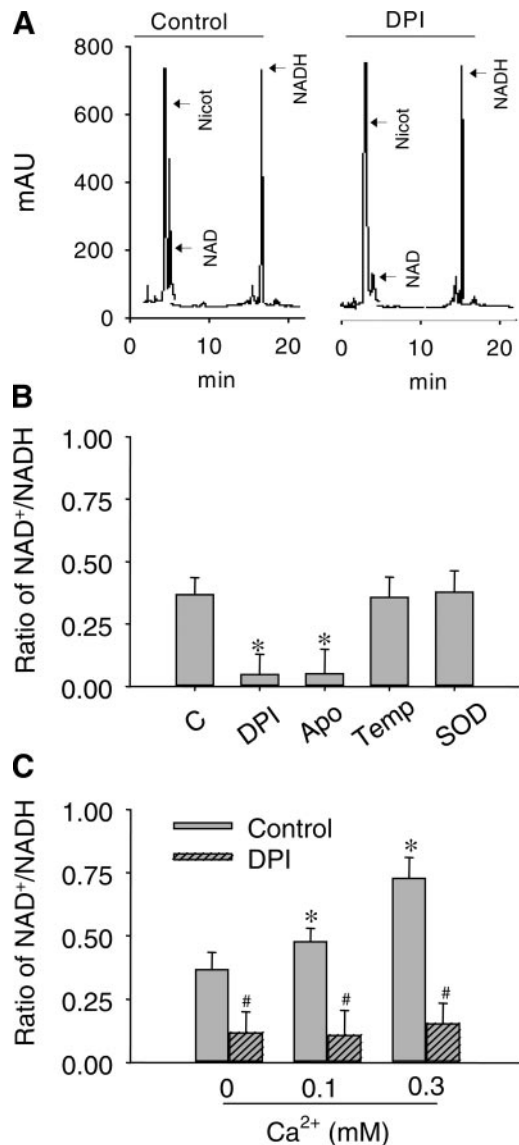


Fig. 3. HPLC analysis of NAD^+ produced by purified SR from bovine CASM. A: typical HPLC chromatogram showing production of NAD^+ detected by a UV array detector. AU, arbitrary units; Nicot, nicotinamide. B: production ratio of NAD^+ to NADH from the SR under control condition and in the presence of NAD(P)H oxidase inhibitors Apo and DPI or SOD and its mimetic tempol (Temp). C: effects of changes in Ca^{2+} concentration on production ratio of NAD^+ to NADH in reaction mixtures with SR and NADH. * $P < 0.05$ compared with 0 mM Ca^{2+} . # $P < 0.05$ compared with control.

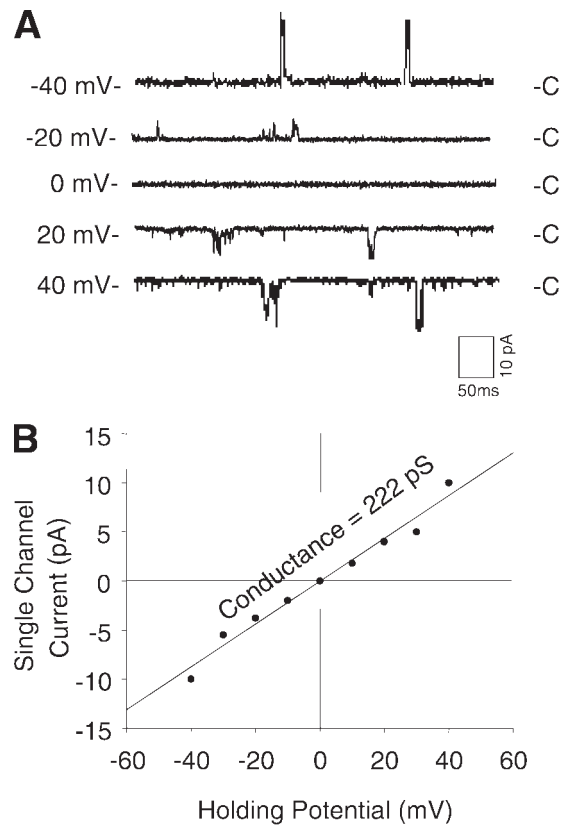


Fig. 4. Characterization of reconstituted ryanodine receptor (RyR)/ Ca^{2+} release channels of purified SR from bovine CASM in the planar lipid bilayer. A: representative recording of ryanodine-sensitive Ca^{2+} channel currents (with Cs^+ as a charge carrier) at holding potential of -40 to $+40$ mV. C, channel closed. Inset: scales of channel recording time (50 ms) and amplitude (10 pA). B: current-voltage relation for reconstituted RyR/ Ca^{2+} release channel currents of SR with symmetrical cesium methanesulfonate (300 mM) solution.

However, SOD and the SOD mimetic tempol had no effect on the conversion of NADH to NAD^+ . On the other hand, addition of $CaCl_2$ to the reaction mixture increased the activity of NAD(P)H oxidase. The ratio of NAD^+ to NADH was increased from 0.370 ± 0.065 to 0.480 ± 0.05 and 0.730 ± 0.08 when $CaCl_2$ with EGTA was added to increase Ca^{2+} levels in the reaction solution to 0.1 and 0.3 mM, respectively. This increase was completely abolished by 50 μ M DPI.

Characterization of Ca^{2+} release channels in the purified SR. Although we have extensive experience in recording RyR/ Ca^{2+} channels in the lipid bilayer reconstituted with the crude coronary arterial SR (27, 45), the characteristics of these channels in the purified SR remain undefined. Therefore, we first examined the biophysical and pharmacological behavior of these channels from the purified SR from coronary arteries. With symmetrical Cs^+ in the *cis* and *trans* solutions, a unitary Cs^+ current through the reconstituted RyR/ Ca^{2+} channel complex in the lipid bilayer was detected at a holding potential of -40 to $+40$ mV (Fig. 4A). The channel amplitude was clearly voltage dependent, with currents increasing in response to increased negative or positive voltages. Mean slope conductance for these Cs^+ currents was 222 ± 15 pS, with reversal potential of 0 mV (Fig. 4B). Ryanodine, a classic RyR activator at low doses, but an inhibitor at high doses, was used to confirm the recorded Cs^+ currents as ryanodine-sensitive Ca^{2+}

release channels. Ryanodine at 0.1–10 μM significantly increased the NP_o of these channels in a concentration-dependent manner with a typical feature of ryanodine-induced subconductance formation. Higher concentrations ($>20 \mu\text{M}$) of ryanodine markedly decreased the NP_o of these channels (Fig. 5, A and B). In contrast, caffeine, a well-known Ca^{2+} -induced Ca^{2+} release activator or RyR stimulator significantly activated these Ca^{2+} release channels. The NP_o was increased from 0.003 ± 0.001 to 0.0128 ± 0.005 in the presence of 5 mM caffeine (Fig. 5, C and D). These results together confirmed that the currents recorded in these purified SR preparations are RyR/ Ca^{2+} release channels.

Effects of NADH on RyR/ Ca^{2+} release channel activity on the SR. Figure 6A shows the representative recordings of RyR/ Ca^{2+} release channels under different conditions. NADH markedly increased the activity of RyR/ Ca^{2+} release channels. In the presence of SOD or tiron, however, NADH-induced activation of these channels was significantly attenuated. As summarized in Fig. 6B, 0.5 mM NADH significantly activated RyR/ Ca^{2+} release channels, with a 4.8-fold increase in the NP_o of these channels. When NADH concentration was increased to 1 mM, the reconstituted bilayer membrane became unstable (i.e., nonspecific leakage current was observed), and the membrane was easily broken, resulting in termination of channel recording in ~ 1 min. Therefore, high concentrations (>1 mM) of NADH might not be tested in this bilayer preparation. In the presence of SOD or tiron, this NADH-induced increase in the NP_o of these channels was obviously blocked. In additional experiments, NAD^+ , a product of NAD(P)H oxidase from

NADH, was tested. It had no effect on the activity of these RyR/ Ca^{2+} channels under control condition or in tiron-treated bilayer preparations (data not shown).

Effects of the NADH oxidase inhibitors on RyR/ Ca^{2+} release channel activity on the SR. The representative recordings depicting the effects of NADH on the activity of SR RyR/ Ca^{2+} release channels in the absence and presence of the NAD(P)H oxidase inhibitors DPI (50 μM), NVN (10 μM), and Apo (100 μM) are presented in Fig. 7A. The activity of RyR/ Ca^{2+} release channels markedly increased when 0.5 mM NADH was added to the *cis* solution, and this NADH-induced activation of the RyR/ Ca^{2+} release channels was blocked by DPI, NVN, and Apo. Figure 7B summarizes the effects of NADH on SR RyR/ Ca^{2+} release channel activity under control condition or in the presence of various NAD(P)H oxidase inhibitors. At 0.5 mM NADH, we observed a 6.4-fold increase in the NP_o of RyR/ Ca^{2+} release channels (0.023 ± 0.007 vs. 0.0036 ± 0.001 in control), which was completely abolished by the NAD(P)H oxidase inhibitors DPI, NVN, and Apo. However, the basal activity of these RyR/ Ca^{2+} release channels was not significantly altered by these NAD(P)H oxidase inhibitors.

Compartment-specific effects of O_2^- on RyR/ Ca^{2+} release channel activity. To identify where exogenously administered O_2^- or NAD(P)H oxidase-derived O_2^- acts to enhance the RyR/ Ca^{2+} channel activity, X/XO, an exogenous O_2^- -producing system, was added to the *cis* or *trans* solution. X/XO activated the RyR/ Ca^{2+} release channels when added to the *cis*, but not the *trans*, side. Figure 8A shows typical recordings

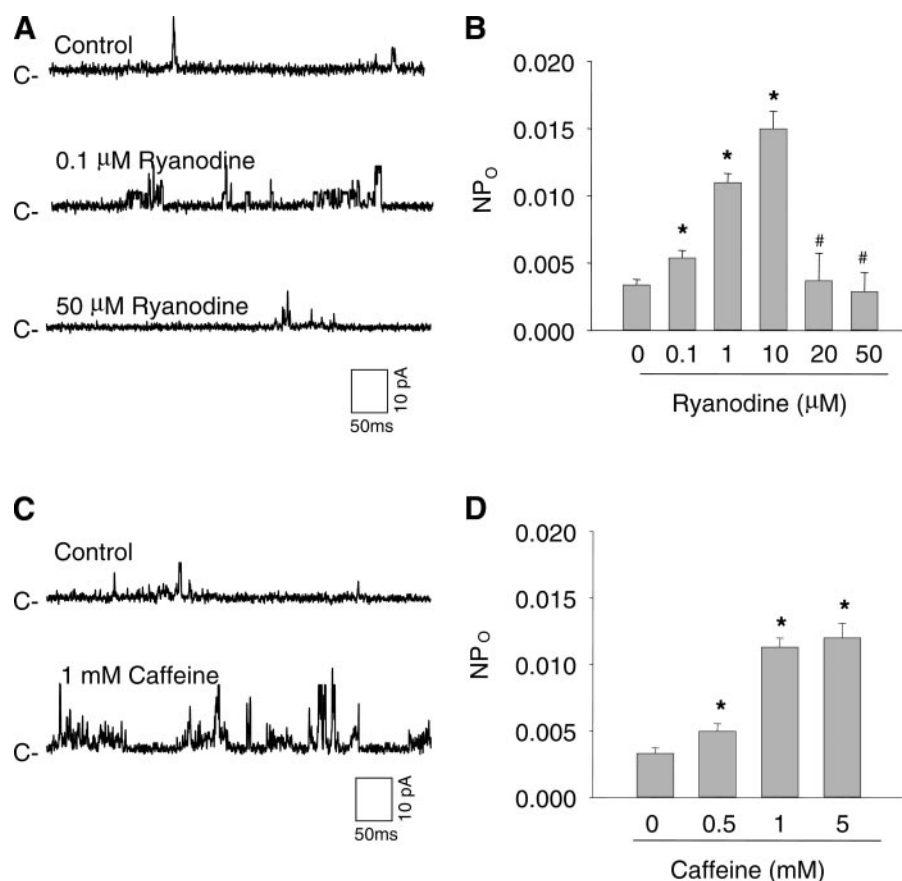


Fig. 5. Effects of ryanodine on reconstituted RyR/ Ca^{2+} release channel activity of purified SR from CASM. A: representative recordings of RyR/ Ca^{2+} channels reconstituted in the planar lipid bilayer without (control) and with ryanodine. B: effects of ryanodine on channel activity. NP_o , open channel probability. C and D: effects of caffeine on RyR/ Ca^{2+} channel activity of purified SR from CASM. * $P < 0.05$ compared with control (0 μM ryanodine/caffeine). # $P < 0.05$ compared with 0.1, 1, or 10 μM ryanodine.

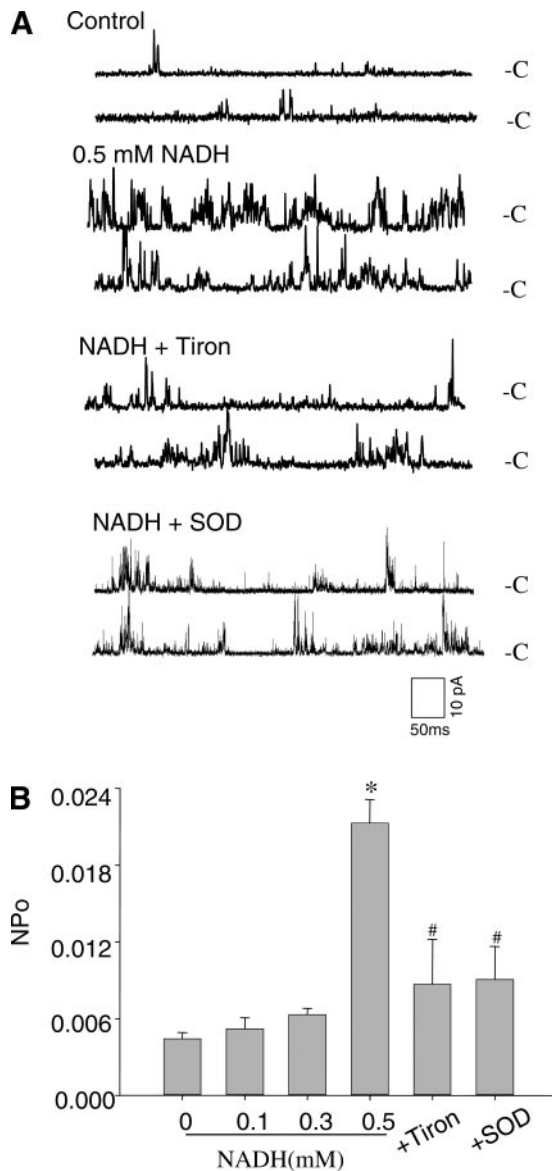


Fig. 6. Effects of NADH on reconstituted RyR/Ca²⁺ release channel activity in purified SR from CASM. A: representative recordings of reconstituted RyR/Ca²⁺ release channels without and with SOD or its chemical mimetic tiron. B: NADH-induced increase in NP_o of RyR/Ca²⁺ release channels. *P < 0.05 compared with control (0 mM NADH). #P < 0.05 compared with 0.5 mM NADH.

of these channels when X/XO was added to the *cis* or *trans* solution. It was clear that only addition of X/XO to the *cis* solution increased the NP_o of RyR/Ca²⁺ channels (Fig. 8B). The NP_o of these RyR/Ca²⁺ channels from bovine CASM was significantly increased when X/XO was added to the *cis* solution, but it was not changed when X/XO was added to the *trans* solution. This *cis*-side-specific effect was also observed when NADH was added. Addition of NADH to the *cis* solution also markedly increased the NP_o of SR RyR/Ca²⁺ release (Fig. 9), but its addition to the *trans* solution had no effect. It seems that O₂^{-•} acts on the *cis* (cytosolic) side of the SR to enhance the activity of SR RyR/Ca²⁺ release channels whether O₂^{-•} is exogenously administered or endogenously produced by the SR NAD(P)H oxidase.

DISCUSSION

It has been demonstrated that, in addition to the interactions of O₂^{-•} and NO in endothelium-dependent vasodilation, the redox signaling mechanism is involved in the constrictor response of arterial smooth muscle to a variety of agonists such as angiotensin II (12, 15, 17), arginine vasopressin (25), nor-epinephrine (44), thromboxane A₂ (46, 58), and endothelin (6, 25). Moreover, endogenous O₂^{-•} has been reported to participate in the vascular myogenic response and the vascular reaction to electrical stimulation (14, 19, 36, 41). Although this redox regulation of vascular function is reportedly attributed to NAD(P)H oxidase-derived O₂^{-•} (6, 20, 23, 31, 42), how this enzyme produces O₂^{-•} and what mechanism mediates the actions of this NAD(P)H oxidase-derived O₂^{-•} in vascular myocytes remain unknown. Fluorescent microscopic imaging analysis and other biochemical assays were used in previous studies to show that O₂^{-•} is accumulated within vascular cells on stimulation, which can be blocked by the NAD(P)H oxidase

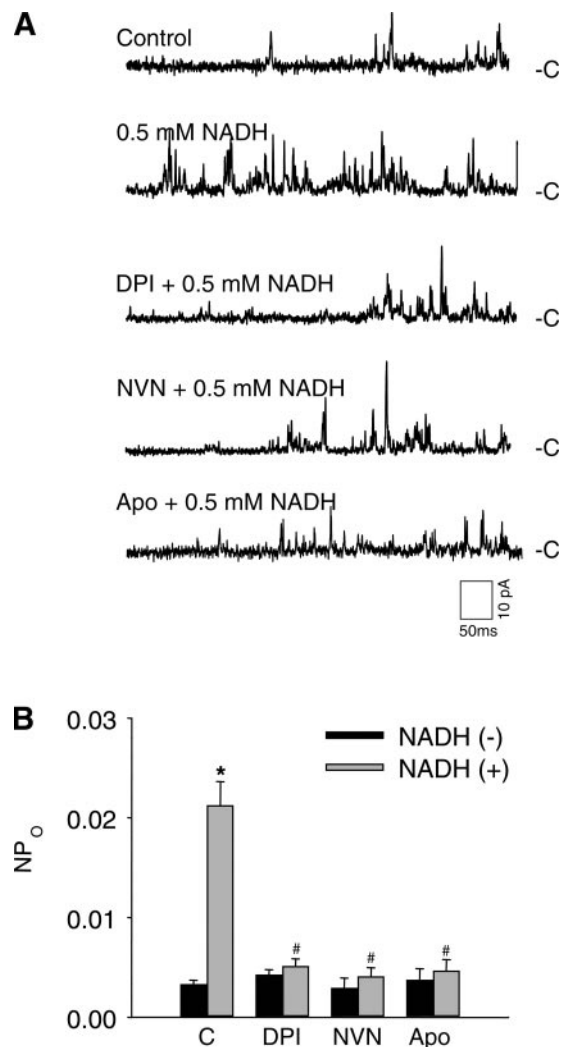


Fig. 7. Effects of NAD(P)H oxidase inhibitors on NADH-induced activation of reconstituted RyR/Ca²⁺ release channels. A: representative recordings of RyR/Ca²⁺ release channel activity without and with DPI, N-vanillylonanamide (NVN), and Apo. B: effects of NAD(P)H oxidase inhibitors on NADH-induced increases in NP_o of reconstituted RyR/Ca²⁺ channels. *P < 0.05 compared with no NADH (-). #P < 0.05 compared with control (C).

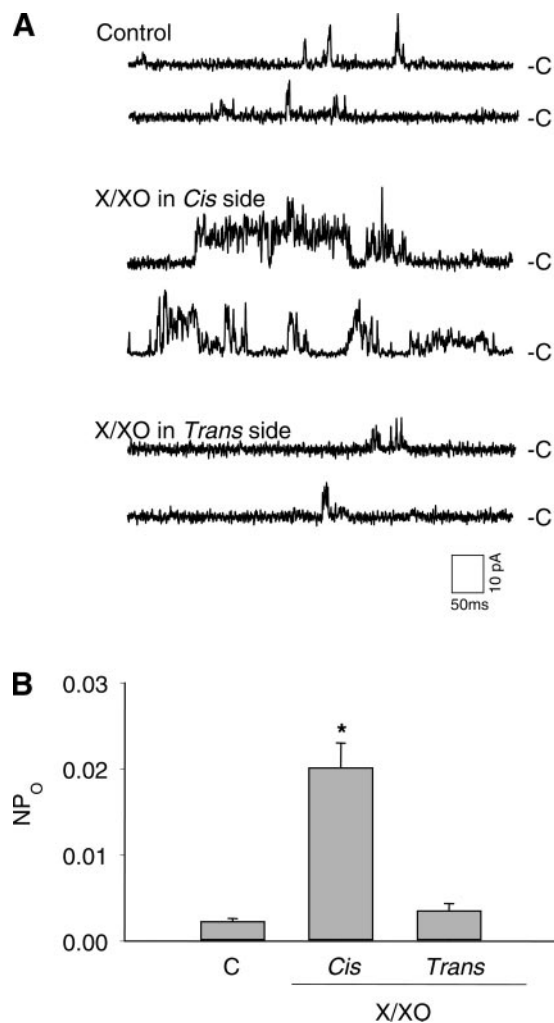


Fig. 8. Effects of xanthine/xanthine oxidase (X/XO) on SR RyR/Ca²⁺ release channel activity reconstituted in the planar lipid bilayer. *A*: representative recordings of RyR/Ca²⁺ release channels when X/XO was added to *cis* or *trans* solution. *B*: effects of X/XO in *cis* or *trans* solution on NP_o of RyR/Ca²⁺ release channels. **P* < 0.05 compared with control (C).

inhibitors (12, 23). The source of this intracellular O₂⁻ is undefined, because the proposed topology of the NAD(P)H oxidase subunits does not indicate that a membrane-associated NAD(P)H oxidase should release O₂⁻ into the cytosol (23), and the subcellular localization analyses of this enzyme in vascular cells have demonstrated that O₂⁻ associated with this non-mitochondrial enzyme within vascular cells may not be derived from plasma membrane (23). It is assumed that some intracellular compartmental NAD(P)H oxidases produce O₂⁻, regulating cell function in vascular myocytes (49, 50). Although they may not reveal how the NAD(P)H oxidase subunits function, the findings of the present study provide direct evidence for the presence of this enzyme on the SR, an intracellular Ca²⁺ store and release organelle.

Using biochemical approaches, we first examined the presence of an NAD(P)H oxidase on the SR of CASM. In the purified SR preparations, Western blot analysis detected four different subunits of NAD(P)H oxidase: Nox4, Nox1, p47^{phox}, and p67^{phox}. However, gp91^{phox} was not detectable in these SR preparations. Because caveolin, a membrane protein marker,

was not detected in these purified SR preparations, it is believed that the NAD(P)H oxidase subunits are not due to contamination from plasma membrane fractions. This SR NAD(P)H oxidase may determine the intracellular O₂⁻ levels in coronary arterial myocytes. In this regard, there is evidence indicating the existence of a cytosolic NAD(P)H system that is functioning to regulate vascular cell functions (49, 50). In addition, a recent study has reported the presence in the skeletal muscle SR of an NADH-dependent oxidase that produces O₂⁻, which activates the SR Ca²⁺ release mechanism in skeletal muscle cells (52). It seems that the presence of the NAD(P)H oxidase in different intracellular compartments such as the cytosol and SR represents one of the important sources of NAD(P)H oxidase-derived O₂⁻.

Next, we examined whether this SR NAD(P)H oxidase in CASM produces O₂⁻. Using a fluorescence spectrometric assay, we determined that the formation rate of ethidium from DHE specific to NADH incubation in the purified SR preparations from CASM represented NAD(P)H oxidase activity. This method was used in previous studies from our laboratory and by others to measure NAD(P)H oxidase activity in different tissues and cells and was confirmed to be specific, reliable, and accurate (26, 33, 56). Time-dependent O₂⁻ production was found in the purified SR preparations from CASM incubated with NADH. Addition of SOD or its chemical mimetic tiron to the reaction abolished formation of the ethidium-DNA complex from DHE, suggesting a blockade of O₂⁻ oxidation of this fluorescent dye. Similarly, when the coronary arterial SR preparations were pretreated with the classical NAD(P)H oxidase inhibitors DPI and Apo, formation of the ethidium-DNA complex from DHE was inhibited. It is clear that the SR purified from CASM is capable of producing O₂⁻.

To further confirm the activity of this SR NAD(P)H oxidase, conversion of NADH to NAD⁺ was also measured by HPLC analysis. Because NAD⁺ is another product in NAD(P)H oxidase-mediated reactions, NAD⁺ formation also reflects the activity of NAD(P)H oxidase in the SR. Consistent with the results from direct assay of O₂⁻ by fluorescence of the ethidium-DNA complex, the production of NAD⁺ as shown by the ratio of NAD⁺ to NADH was significantly increased when the purified SR was incubated with NADH. Blockade of this conversion of NADH to NAD⁺ by DPI or Apo suggests that the NAD(P)H oxidase reaction mediates production of NAD⁺ in the SR. Because NAD(P)H oxidase was reported to be a

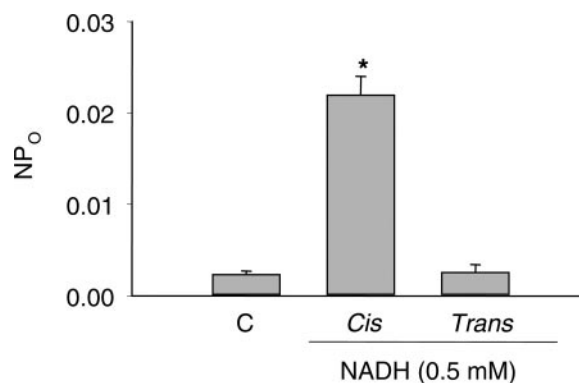


Fig. 9. Effects of NADH in *cis* or *trans* solution on NP_o of SR RyR/Ca²⁺ release channels reconstituted in the planar lipid bilayer. **P* < 0.05 vs. control (C).

Ca²⁺-dependent enzyme (3, 24), we also determined the effects of different Ca²⁺ concentrations on the conversion of NADH to NAD⁺. It seems that this SR NAD(P)H oxidase is also Ca²⁺ sensitive, because conversion of NADH to NAD⁺ significantly increased when Ca²⁺ concentrations in the SR NADH reaction mixture were increased. It is assumed that this Ca²⁺ sensitivity of the SR NAD(P)H oxidase may be important in the regulation of intracellular O₂⁻ levels in CASM cells. In this regard, Ca²⁺ released from the SR or other sources enhances O₂⁻ production by SR NAD(P)H oxidase, which may contribute to the increase in O₂⁻ levels in response to different agonists such as angiotensin II (12, 15, 17), arginine vasopressin (25), norepinephrine (44), thromboxane A₂ (46, 58), and endothelin (6, 25), as well as direct stretch and electrical stimulations to the vessel wall (14, 19, 36, 41). It is possible that these stimuli first increase intracellular Ca²⁺ and, thereby, activate NAD(P)H oxidase on the SR, resulting in a global increase in intracellular O₂⁻ levels. This may be one of the important mechanisms mediating the redox response of the arteries to various agonists and stretch and electrical stimuli.

To explore the physiological relevance of this SR NAD(P)H oxidase, we examined the effects of O₂⁻ production by this enzyme on the activity of SR RyR Ca²⁺ release channels by reconstituting these channels into the lipid planar bilayer. The purified SR from CASM was used to record and characterize these SR RyR/Ca²⁺ release channels, the characteristics of which were observed in our previous studies in which crude SR preparations were used (27, 45). Interestingly, when NADH was added to the *cis* side of the bilayer, the activity of reconstituted RyR/Ca²⁺ release channels from the purified SR of CASM was significantly enhanced. However, NAD⁺ had no effect on the activity of these channels. In the presence of SOD or its mimetic tiron, this NADH-induced activation of RyR/Ca²⁺ release channels was significantly attenuated. It seems that NAD(P)H oxidases incorporated into the planar lipid bilayer or present in the bath can use NADH as a substrate to produce O₂⁻ and, thereby, activate RyR/Ca²⁺ channels. This view was also supported by the findings that NADH-induced activation of the SR RyR/Ca²⁺ channels could be blocked by various NAD(P)H oxidase inhibitors such as DPI, Apo, and NVN, which act to inhibit NAD(P)H oxidase by different mechanisms. These results provide evidence that SR NAD(P)H oxidase-derived O₂⁻ locally participates in regulation of RyR/Ca²⁺ channel activity. This may represent an important mechanism responsible for the redox regulation of intracellular Ca²⁺ movements and consequent vasomotor response of arterial smooth muscle to different stimuli (see above).

Activation of RyR by O₂⁻ has been extensively studied (4, 5, 8, 9, 37). A thiol-disulfide exchange model in cardiac and skeletal muscle has been proposed to describe the mechanism by which O₂⁻ directly activates the RyR/Ca²⁺ release on the SR (2, 11, 22, 35, 51, 55). In this model, intramolecular thiol-disulfide interexchange reactions within RyR control open or closed states of its Ca²⁺ release channels (10, 18, 32, 48, 54). When the thiol groups of RyR/Ca²⁺ release channels are in a reduced status (-SH form), the channels are closed. In contrast, the channels are open when disulfide is formed by oxidation of thiol groups of RyR (-S-S- form) (5, 7, 8, 43). The present study did not repeat these experiments in CASM but, rather, examined localization of the O₂⁻ action on the RyR/Ca²⁺ release channels on the SR reconstituted in the planar

lipid bilayer. By adding a classical O₂⁻-producing enzyme system, X/XO, to the *cis* and *trans* solutions, we found that only on the *cis* side was O₂⁻ produced from this enzyme system able to activate the RyR/Ca²⁺ channels. Similarly, NADH increased the activity RyR/Ca²⁺ release channels only when added to the *cis* solution. Because the *cis* side of the lipid bilayer represents the cytosol compartment, these results suggest that activation of the SR RyR/Ca²⁺ release channels by O₂⁻ is an response to increases in its intracellular levels in the cytoplasm, whether O₂⁻ is derived from the SR NAD(P)H oxidase or from other sources.

In summary, the present study demonstrated that an NAD(P)H oxidase is functioning on the SR of CASM and that O₂⁻ derived from this NAD(P)H oxidase locally activates the RyR/Ca²⁺ release channels on the SR to mobilize Ca²⁺. Given the Ca²⁺ sensitivity of this SR NAD(P)H oxidase, increases in intracellular Ca²⁺ levels would further activate or enhance the activity of this enzyme, thereby forming a positive-feedback or amplifying pathway in the regulation of intracellular Ca²⁺ levels. This redox-amplifying mechanism of Ca²⁺ signaling has yet to be clarified in detail.

GRANTS

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