Nitric oxide affects sarcoplasmic calcium release in skeletal myotubes

LEO M. A. HEUNKS,1 HERWIN A. MACHIELS,1 P. N. RICHARD DEKHUIJZEN,1
Y. S. PRAKASH,2 AND GARY C. SIECK2,3

1Department of Pulmonary Diseases, University Hospital Nijmegen,
Nijmegen, the Netherlands; 2Departments of Anesthesiology and 3Physiology
and Biophysics, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Received 16 March 2000; accepted in final form 16 July 2001

Nitric oxide affects sarcoplasmic calcium release in skeletal myotubes. J Appl Physiol 91: 2117–2124, 2001.—In the present study, we used real-time confocal microscopy to examine the effects of two nitric oxide (NO) donors on acetylcholine (ACh; 10 μM)- and caffeine (10 mM)-induced intracellular calcium concentration ([Ca2+]i) responses in C2C12 mouse skeletal myotubes. We hypothesized that NO reduces [Ca2+]i in activated skeletal myotubes through oxidation of thiols associated with the sarcoplasmic reticulum Ca2+-release channel. Exposure to diethylamine NONOate (DEA-NO) reversibly increased resting [Ca2+]i, level and resulted in a dose-dependent reduction in the amplitude of ACh-induced [Ca2+]i responses (25 ± 7% reduction with 10 μM DEA-NO and 72 ± 14% reduction with 100 μM DEA-NO). These effects of DEA-NO were partly reversible after subsequent exposure to dithiothreitol (10 mM). Preexposure to DEA-NO (1, 10, and 50 μM) also reduced the amplitude of the caffeine-induced [Ca2+]i response. Similar data were obtained by using the chemically distinct NO donor S-nitroso-N-acetyl-penicillamine (100 μM). These results indicate that NO reduces sarcoplasmic reticulum Ca2+-release in skeletal myotubes, probably by a modification of hyperreactive thiols present on the ryanodine receptor channel.

ryanodine receptor; fluorescence; C2C12 myotubes

NITRIC OXIDE (NO) MODULATES in vitro contractility of skeletal muscle (12, 20, 23). For instance, exposing rat diaphragm muscle strips to the NO donor sodium nitroprusside reduces submaximal force generation (12). However, the specific mechanisms by which NO modifies skeletal muscle contractility are not as yet known. NO synthase (NOS) is the physiological source for NO, and NOS expression has been confirmed in rodent and human skeletal muscle fibers (12, 13, 22). Generation of NO by skeletal muscles is enhanced by contractile activity (4, 12). The inhibitory effects of NO on force production could result from interference of NO with intracellular Ca2+ concentration ([Ca2+]i) regulation. One potential target is the sarcoplasmic reticulum (SR). For instance, it has been shown that the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) reduces caffeine-induced Ca2+ release in isolated skeletal muscle SR vesicles (18). However, in intact mouse skeletal muscle fibers, SNAP increased [Ca2+], during submaximal activation without altering force generation (3). Previous studies have shown that NO reduces [Ca2+]i in skeletal muscle SR vesicles (18, 26, 28, 30). Previous studies in skeletal muscle SR vesicles indicated that NO increases the open probability of the RyR channel in the absence of a RyR agonist (26). However, other studies suggest that NO reduces RyR channel-mediated Ca2+ release in caffeine-activated vesicles (18). Few studies have been performed on the effects of NO on skeletal muscle fibers, SNAP increased [Ca2+], responses during submaximal activation (3). However, it was uncertain whether this was the result of increased RyR channel-mediated Ca2+ release or impaired Ca2+ reuptake.

The purpose of the present study was to investigate the effects of NO on SR Ca2+ release in intact skeletal muscle cells. We measured [Ca2+]i responses by using real-time confocal imaging of fluo 3-loaded C2C12 skeletal myotubes, which were activated by acetylcholine (ACh) and the RyR agonist caffeine. Myotubes were exposed to two different NO donors at varying concentrations. To test the possible involvement of sulfhydryl oxidation, myotubes were exposed to the reducing agent 1,4-dithiothreitol (DTT) after exposure to NO donors. We hypothesized that NO reduces [Ca2+]i in activated skeletal myotubes through oxidation of thiols associated with the SR RyR Ca2+ release channel.

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METHODS

Cell Culture

C₂C₁₂ myoblasts (American Type Culture Collection, Manassas, VA) were grown on 25-mm glass coverslips (37°C, 5% CO₂) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). The medium also contained 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. When cells reached confluence, FBS was substituted by 10% horse serum (Gibco) to enhance differentiation into myotubes. Experiments were performed 4–6 days after serum replacement and within 10 days. Under our experimental conditions, spontaneous contractions were observed sporadically and were used as an index of myotube differentiation. All sets of experiments were performed on myotubes from at least two different passages.

Intracellular Ca²⁺ Imaging

Cytoplasmic free Ca²⁺ levels were determined by fluo 3 fluorescence. Plated myotubes were incubated in 5 μM fluo 3-AM (Molecular Probes, Eugene, OR) in Hanks' balanced salt solution (HBSS) at room temperature for 45 min and subsequently washed with HBSS. The coverslip was placed in an open slide chamber (Warner Instruments, Hamden, CT) mounted on a Nikon Diaphot inverted microscope. The chamber was perfused with HBSS at 1–2 ml/min at room temperature.

Details on the techniques for real-time confocal imaging of [Ca²⁺], have been previously described (24). Briefly, an Odyssey XL real-time confocal microscope (Noran Instruments, Middleton, WI) with an Ar-Kr laser and mounted on a Nikon Diaphot inverted microscope was used to visualize fluo 3-AM (Molecular Probes)-loaded myotubes. The confocal system was controlled through a Silicon Graphics Indy workstation and manufacturer-supplied software. A Nikon ×40/1.3 oil-immersion objective lens was used to visualize the myotubes. Image size was set at 640 × 480 pixels, and the pixel area was calibrated by using a standard micrometer (0.063 μm²/pixel). The 488-nm laser line was used to excite fluo 3 for calcium imaging, and emissions were collected using a 515-nm long-pass filter and high-sensitivity photomultiplier tube. On the basis of preliminary studies, laser intensity and photomultiplier gain were set to ensure that pixel intensities were within 25 and 255 gray levels. To minimize bleaching, laser exposure time was kept as short as possible. The Odyssey confocal system is capable of acquiring 480 frames/s. However, we found that an acquisition rate of 30 frames/s was sufficient for measuring amplitudes of [Ca²⁺], responses. Eight regions of interest (ROIs) of 5 × 5 pixels were defined before each experiment. The amplitude and rise time of each [Ca²⁺], transient were measured (see Fig. 1 for definitions). Rise time was normalized for differences in amplitude.

Selection of Myotubes

At confluence, the coverslip was typically dense with myotubes and undifferentiated cells. Only large, intact myotubes were used for [Ca²⁺], measurements. Usually it was possible to visualize two to three myotubes per image field. Usually two to four ROIs were defined within each myotube; however, [Ca²⁺], measurements from only one of these ROIs (typically the ROI displaying the largest amplitude response) was used to represent each myotube during the remaining experimental protocol. In general, cells from one coverslip were used for only one experimental protocol. Occasionally, myotubes became partially detached from the coverslip during the experiment. Data from these myotubes were omitted from subsequent analysis.

Ca²⁺ Regulation in Myotubes

Effect of ACh on [Ca²⁺]. To determine the maximal concentration for ACh to stimulate myotubes, the dose-response relationship was evaluated. After measurement of [Ca²⁺],

Fig. 1. Schematic representation of acetylcholine (ACh)-induced intracellular calcium concentration ([Ca²⁺]), transients in the absence of extracellular Ca²⁺, in a fluo 3-loaded C₂C₁₂ skeletal myotube. The amplitude of the transients, expressed as gray levels (GL), was not uniform among the myotube.
under resting conditions, myotubes were sequentially exposed to a range of ACh concentrations (10, 100, and 1,000 μM). To ensure optimal refilling of the internal Ca^{2+} stores, the tissue chamber was perfused with standard HBSS for 10 min between different ACh concentrations. From these experiments, it appeared that a maximal [Ca^{2+}]_{i} response was acquired at 10 μM ACh. Therefore, in subsequent experiments, a 10 μM ACh concentration was used.

**Effect of extracellular Ca^{2+} on ACh-induced [Ca^{2+}]_{i} responses.** ACh-induced [Ca^{2+}]_{i} responses in C2C12 myotubes may partly result from Ca^{2+} influx (7). Furthermore, Ca^{2+} influx may also induce Ca^{2+}-induced Ca^{2+} release. To determine the contribution of Ca^{2+} influx in our preparation, myotubes were successively exposed to ACh in standard HBSS and “zero-Ca^{2+}” HBSS. Before ACh exposure in zero-Ca^{2+} HBSS, extracellular Ca^{2+} was washed out for 60 s with zero-Ca^{2+} HBSS. Zero-Ca^{2+} HBSS had similar composition as standard HBSS, except that 2.0 mM CaCl_{2} was substituted with 2.0 mM EGTA. In time controls, both ACh stimulations were conducted in the presence of extracellular Ca^{2+}.

In accordance with previous studies (6), it appeared that the ACh-induced [Ca^{2+}]_{i} response was, in fact, partly due to Ca^{2+} influx (see RESULTS). Because the focus of the present study was to investigate the effects of NO on SR Ca^{2+} release, all subsequent experiments evaluated ACh-induced [Ca^{2+}]_{i} responses in the absence of extracellular Ca^{2+}. However, to ensure optimal reloading of intracellular Ca^{2+} stores, myotubes were exposed to standard HBSS between all ACh stimulations. Before the exposure to ACh, the tissue chamber was perfused with zero-Ca^{2+} HBSS for 1 min to wash out extracellular Ca^{2+}.

**Effects of ryanodine and Xestospongin D on [Ca^{2+}]_{i} responses.** In C2C12 myotubes, ACh-induced SR Ca^{2+} release may be mediated via both ryanodine and inositol 1,4,5-trisphosphate (IP_{3}) sensitive stores (7). To determine the relative contribution of each of these SR release channels, we evaluated ACh-induced [Ca^{2+}]_{i} responses in the presence of high concentrations of ryanodine (100 μM) to specifically block the RyR channel and in the presence of the membrane permeant IP_{3} receptor (IP_{3}R) channel blocker Xestospongin D (XeD, 10 μM) (5). Stock solutions of XeD (Calbiochem) were kept frozen, and just before use, XeD was thawed and subsequently diluted to 10 μM with HBSS. This XeD concentration was chosen because it had been shown to effectively block IP_{3}R without exhibiting significant effects on RyR-mediated SR Ca^{2+} release (5).

After measurement of the initial ACh-induced [Ca^{2+}]_{i} response, myotubes were washed, then exposed to 100 μM ryanodine for 5 min and subsequently reexposed to ACh. In a second set of experiments, the effect of 10 μM XeD was determined in a similar protocol. In a third set of experiments, the effect of ryanodine on caffeine-induced [Ca^{2+}]_{i}, responses was studied.

**NO and Ca^{2+} Regulation in Myotubes**

**Effect of DEA-NO on ACh-induced [Ca^{2+}]_{i}, transients.** After measurement of ACh-induced [Ca^{2+}]_{i} transients, myotubes were exposed to standard HBSS for 5 min to ensure optimal reloading of internal Ca^{2+} stores. Subsequently, myotubes were exposed to one concentration of diethylamine NONOate (DEA-NO; 10, 50 or 100 μM, Calbiochem) for 10 min before a second ACh stimulation. The intracellular stores were then reloaded, and myotubes were exposed to the reduc tant DTT before a final ACh stimulation in the continued presence of DTT.

**Effect of DEA-NO on caffeine-induced [Ca^{2+}]_{i}, transients.** To determine whether the effects of DEA-NO on ACh-induced [Ca^{2+}]_{i} responses were the result of modification of the RyR Ca^{2+} channel, experiments with DEA-NO were repeated by using the RyR agonist caffeine (10 mM) instead of ACh. Because 50 and 100 μM DEA-NO had similar effects on ACh-induced [Ca^{2+}]_{i} responses (see RESULTS), lower concentrations of DEA-NO (1, 10, and 50 μM) were used to study the effect of NO on caffeine-induced [Ca^{2+}]_{i} release. A similar protocol was used for time controls except that DEA-NO was omitted from HBSS solutions.

**Effect of SNAP on caffeine-induced [Ca^{2+}]_{i}, transients.** Theoretically, the effects of DEA-NO on [Ca^{2+}]_{i} responses could be the result of a NO-independent action of this donor. To rule out this possibility, the caffeine experiments were repeated by using the chemically distinct NO donor SNAP (100 μM) instead of DEA-NO.

**Statistics**

The effects of NO donors on the amplitude of ACh and caffeine [Ca^{2+}]_{i} responses were analyzed with SPSS/PC+, version 9.0. (SPSS, Chicago, IL). Results are expressed as means ± SE. To compare independent groups (e.g., ACh responses in time control and ryanodine-treated myotubes), Mann-Whitney's rank sum test was used. For two related events (i.e., ACh response in XeD compared with subsequent exposure to ryanodine), Wilcoxon's test was used. Significance was set at P < 0.05. The numbers of myotubes are given with the results for each experimental protocol.

**RESULTS**

**General Characteristics**

Myotubes exposed to either ACh or caffeine in the absence of extracellular Ca^{2+} exhibited no diffuse elevation in [Ca^{2+}]_{i}, but rather focal areas of high responsiveness (Fig. 1). This heterogeneity has been recognized previously (6, 9) and is thought to be the result of a heterogeneous distribution of SR (9). In addition, both baseline fluorescence and the absolute height of the amplitude (in gray levels) varied among myotubes because of differences in uptake of fluo 3-AM. Therefore, effects of experimental procedures were determined by measuring relative changes in the amplitude of [Ca^{2+}]_{i}, transients (see METHODS).

**Effect of extracellular Ca^{2+} on ACh-induced [Ca^{2+}]_{i}, transients.** Omission of Ca^{2+} from the extracellular medium altered the shape and reduced the amplitude of ACh-induced [Ca^{2+}]_{i} transients (see METHODS). Exposing myotubes to ACh in the presence of extracellular Ca^{2+} resulted in a rapid and sustained elevation of [Ca^{2+}]_{i}. However, when extracellular Ca^{2+} was omitted, ACh induced a rapid monophasic response (Fig. 2). The amplitude of this latter response was 62.5 ± 4.6% of the response in the presence of Ca^{2+} compared with 98.2 ± 9.8% in time controls (n = 10; P < 0.05 vs. time control). However, omission of extracellular Ca^{2+} did not affect rise time and/or amplitude of the transient (66 ± 11 in the presence of extracellular Ca^{2+} vs. 99 ± 25% in the absence of extracellular Ca^{2+}; P > 0.05).

**Effects of ryanodine and XeD on [Ca^{2+}]_{i}, transients.** Exposure to 100 μM ryanodine resulted in a significant reduction in the amplitude of ACh-induced [Ca^{2+}]_{i},

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*J Appl Physiol* • VOL 91 • NOVEMBER 2001 • www.jap.org
Table 1. Effect of DEA-NO on baseline fluorescence in skeletal myotubes

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<tr>
<th>DEA-NO</th>
<th>DTT</th>
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<tr>
<td>Time control</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>DEA-NO, 10 μM</td>
<td>106 ± 3*</td>
</tr>
<tr>
<td>DEA-NO, 50 μM</td>
<td>106 ± 3*</td>
</tr>
<tr>
<td>DEA-NO, 100 μM</td>
<td>102 ± 5</td>
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Values are mean percentages of initial baseline fluorescence ± SE. DEA-NO, diethylamine NONOate; DTT, dithiothreitol. *Significantly different from time control (P < 0.05).

Effect of DEA-NO on amplitude of ACh-induced [Ca2+]i transients. Exposure to DEA-NO resulted in a reduction in the amplitude of [Ca2+]i responses to ACh but did not affect the rise time of the responses (Table 2). A representative trace is shown in Fig. 3A. In myotubes exposed to 10-μM DEA-NO, amplitude of the transient (n = 11) was 75 ± 7% of time control (n = 13; Fig. 3B). Exposure to 50 or 100 μM DEA-NO further reduced the amplitude of the [Ca2+]i response (n = 11 and n = 9, respectively). Subsequent exposure to DTT reversed the effects of 50 and 100 μM DEA-NO. However, DTT did not reverse the effects of 10 μM DEA-NO in all fibers.

Effect of DEA-NO on amplitude of caffeine-induced [Ca2+]i transients. Exposure to 1.0 μM DEA-NO did not affect the amplitude of the caffeine-induced [Ca2+]i responses (n = 4). However, the amplitude of caffeine-induced [Ca2+]i responses was significantly reduced and the rise time significantly increased after exposure to either 10 or 50 μM DEA-NO (n = 9 and n = 6, respectively; P < 0.05). After exposure to DTT, amplitude of the transient was 92 ± 20 and 88 ± 27% of baseline amplitude, respectively. In time controls, amplitude of the third [Ca2+]i response was 128 ± 8.7% of initial response (n = 12; Fig. 4).

Table 2. Effect of DEA-NO on rise time/amplitude in skeletal myotubes

<table>
<thead>
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<th>Preexposure</th>
<th>Exposure</th>
<th>Postexposure</th>
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<tr>
<td>DEA-NO, 10 μM (ACh)</td>
<td>11.3 ± 1.8</td>
<td>18.9 ± 3.5</td>
<td>15.6 ± 4.4</td>
</tr>
<tr>
<td>DEA-NO, 10 μM (Caf)</td>
<td>114 ± 23</td>
<td>635 ± 21*</td>
<td>179 ± 48</td>
</tr>
</tbody>
</table>

Values are means ± SE (in ms/amplitude). Other concentrations for ACh-induced intracellular Ca2+ concentration transients are not shown because, in many myotubes, DEA-NO completely inhibited the transient so no rise time could be calculated. ACh, acetylcholine; Caf, caffeine. *Significantly different compared with preexposure (P < 0.05).
versed the effects of SNAP (92.5 ± 12.0% of initial amplitude; P < 0.05 compared with time controls).

**DISCUSSION**

The present study demonstrates that DEA-NO decreases the amplitude of ACh-induced [Ca^{2+}]_i transients in mouse skeletal myotubes. The effects of DEA-NO were partly reversible after exposure to DTT, indicating that thiol modification contributes to these effects. The fact that both DEA-NO and the chemically distinct NO donor SNAP have similar effects on caffeine-induced [Ca^{2+}]_i transients suggests that the reduction in the height of amplitude of the [Ca^{2+}]_i transients result from thiol modification of the RyR Ca^{2+}-release channel.

**Cultured Myotubes**

C_{2}C_{12} mouse skeletal myotubes were used to study the effects of NO on SR Ca^{2+} release. A potential issue with such a preparation is whether these findings are also applicable to mature skeletal muscles. C_{2}C_{12} myotubes reach a considerable degree of differentiation as indicated by the expression of fast-twitch skeletal muscle troponin T, α-actin, and tropomyosin (16). Permeabilized myotubes generate measurable force when perfused with Ca^{2+}, and the concentration needed to generate 50% of maximal force was within the range of mature rat skeletal muscle fibers (16). In the present study, we purposely selected myotubes that were clearly differentiated as indicated by the presence of at least some sarcomere pattern.

Mechanisms of [Ca^{2+}]_i handling in C_{2}C_{12} myotubes have been investigated in detail by other laboratories (6–10). Two striking differences in [Ca^{2+}]_i regulation between mature skeletal muscle fibers and myotubes have been shown. First, elevation of [Ca^{2+}]_i on stimulation with ACh is partly the result of Ca^{2+} influx through voltage-gated Ca^{2+} channels, whereas in mature skeletal muscle fibers, the contribution of Ca^{2+} influx during activation is considered to be of limited significance. Indeed, we found that omission of Ca^{2+} from the extracellular medium altered the shape and reduced the amplitude of ACh-induced [Ca^{2+}]_i transients. To circumvent the contribution of Ca^{2+} influx during activation is considered to be of limited significance. Indeed, we found that omission of Ca^{2+} from the extracellular medium altered the shape and reduced the amplitude of ACh-induced [Ca^{2+}]_i transients.
pathway is considered to be of limited relevance in mature skeletal muscle (17). In our culture preparation, we found a significant contribution of IP_{3}-sensitive stores in ACh-induced [Ca^{2+}]_{i} transients. When myotubes were preexposed to XeD, which is a membrane-permeable noncompetitive IP_{3}R blocker (5), amplitudes of transients elicited by ACh were attenuated by ~58%. On the other hand, ryanodine reduced the amplitude of ACh-induced [Ca^{2+}]_{i} transients by ~50%. This indicates that IP_{3} and RyR Ca^{2+} release channels have about equal contribution to ACh-induced elevation in [Ca^{2+}]_{i} in skeletal myotubes. As expected, XeD did not affect caffeine-induced Ca^{2+} transients. To omit contribution of Ca^{2+} release via IP_{3}R, additional experiments were performed with the RyR agonist caffeine.

Fluorescence Signals

Changes in [Ca^{2+}]_{i} were monitored by using fluo 3, which is a nonratiometric dye. To obtain an estimate of the actual [Ca^{2+}]_{i}, a calibration procedure should be performed that requires permeabilization of the cells. However, because the myotubes in our preparation were quite delicate, such procedures were impractical, a problem that has been recognized by other groups by using C_{2}C_{12} myotubes as well (6, 10). Nevertheless, relative changes in the amplitude of fluorescence signals because of experimental procedures (e.g., DEA-NO) provide (semiquantitative) information on the effects of such procedures on [Ca^{2+}]_{i} handling. The lack of significant differences in [Ca^{2+}]_{i} responses of time controls indicates that the results with DEA-NO cannot be explained by loss of cell viability.

NO donors. Because NO in its pure form is highly reactive and has limited solubility in aqueous buffers, it is difficult to predictably deliver it into biological systems without decomposition. Therefore, NO donors are frequently used to study the (patho)physiological effects of NO on tissue function. However, a perennial question with the use of NO donors is the validity of equating the donor to the production of NO. Numerous chemically distinct NO donors are commercially available. We chose DEA-NO, which belongs to the group of NO-nucleophile adducts. This type of donor, with the general structure (XN(O)NO)^{-}, spontaneously releases NO (19). The rate of release is only dependent on pH, temperature, and the structure of the nucleophilic residue (19). It is easy to control the initiation of the release of NO from DEA-NO by adjusting pH and temperature of the stock solution. To verify that the observed effects were mediated via NO, part of the experiments were repeated using the chemically distinct NO donor SNAP. In addition, previous studies have shown that parent compounds for DEA-NO (up to 300 μM) and breakdown products do not affect force generation in smooth muscle (11).

Mechanisms of NO Action

It has been shown that thiol groups present on the RyR Ca^{2+} release channel play a role in the regulation of its open probability (1). NO can react with thiol groups via either S-nitrosylation or by influencing disulphide formation (25). Therefore, the RyR Ca^{2+} release channel is a possible target for NO. The present study supports the hypothesis that NO may modulate SR Ca^{2+} release by modulating thiol groups in intact cells. DEA-NO had dose-dependent effects on ACh-induced [Ca^{2+}]_{i} responses, and these effects were partly reversible after exposure to the reducing agent DTT. Although ACh in vivo initiates the excitation-contraction coupling cascade, these data do not indisputably indicate that the RyR Ca^{2+}-release channel was the primary target for NO because other proteins involved in excitation-contraction coupling contain thiol groups as well. Therefore, experiments were repeated using the RyR agonist caffeine. Data from these latter experiments showed that two distinct NO donors reversibly decreased the amplitude of [Ca^{2+}]_{i} transients. This indicates that the effects of NO on [Ca^{2+}]_{i} transients are mediated by modification of thiols on the SR RyR Ca^{2+} release channel. Data from the present study are in line with observations from previous studies that used isolated SR vesicles. Exposing SR vesicles to SNAP has been found to reduce the rate of caffeine-induced Ca^{2+} release (18). In addition, the open probability of the RyR Ca^{2+} release channel in lipid bilayers is reduced after exposure to 100 μM SNAP (18), which is a similar concentration to that used in the present study. Decreased open probability of the channel after exposure to NO may be the result of the inhibitory effect of NO on intersubunit cross-linking of thiol groups on the RyR channel, thereby preventing oxidant-induced activation of the channel (2). In contrast, at high concentration, SNAP increases RyR Ca^{2+} release channel open probability (2, 26), indicating differential concentration-dependent effects of NO.

Reduced [Ca^{2+}]_{i} transients after preexposure to NO donors may also result from increased SR Ca^{2+} ATPase activity. Although we could not definitely exclude this possibility, it is an unlikely explanation. Studies using SR vesicles indicated that NO donors did not affect (18) or even inhibit Ca^{2+} reuptake (26). Furthermore, in intact skeletal muscle fibers, Ca^{2+} uptake was decreased because of treatment with NO donors (3). In the present study, we studied the effects of NO donors only on the amplitude of [Ca^{2+}]_{i} transients. Maximal amplitude is reached quickly after the initiation of the transient (Fig. 1) and is, therefore, primarily dependent on the rate of SR Ca^{2+} release. In contrast, the height of the plateau after maximal amplitude is determined by the equilibrium between Ca^{2+} release and reuptake in the SR and efflux from the myoplasm. Together, it is unlikely that the effects of NO donors on the amplitude of [Ca^{2+}]_{i} transients arise from an increase in SR Ca^{2+} ATPase activity.

Baseline [Ca^{2+}]_{i} levels were elevated after exposure to DEA-NO. This is in line with studies on intact mouse single fibers (3) and may be the result of inhibition of Ca^{2+} ATPase activity (3, 26) or increased Ca^{2+} leak from the SR (26). It is tempting to speculate whether a direct relation exists between NO donor-induced eleva-
tion in baseline [Ca\(^{2+}\)], and the reduction in the amplitude of ACh- and caffeine-induced [Ca\(^{2+}\)] transients. In theory, increased Ca\(^{2+}\) leakage through RyR Ca\(^{2+}\) release channels may partly depolarize the SR, which, in turn, might reduce the amplitude of ACh- and caffeine-induced Ca\(^{2+}\) concentration transients. The relationship between luminal SR Ca\(^{2+}\) and RyR Ca\(^{2+}\) release channel function has been the subject of several recent studies. For instance, it has been shown that an inverse correlation exists between SR luminal Ca\(^{2+}\) concentration and open probability of the RyR Ca\(^{2+}\) release channel (27). However, in skinned skeletal muscle fibers, the apparent rate constant of caffeine-induced SR Ca\(^{2+}\) efflux did not significantly change when the SR loading level was decreased to one-third of maximal loading (14). Although studying the effects of NO donors on SR luminal Ca\(^{2+}\) was beyond the scope of the present study, we do not believe that depletion of SR luminal Ca\(^{2+}\) explains the NO donors mediated reduction in ACh and caffeine-induced [Ca\(^{2+}\)] amplitudes. First, between subsequent stimulations, myotubes were incubated in HBSS containing 2.0 mM Ca\(^{2+}\) for 10 min to refill SR. Second, studies on single RyR Ca\(^{2+}\) release channels support a direct role for NO donors in decreasing the open probability of these channels (18).

Our data are in apparent conflict with a recent report by Andrade et al. (3), which found that exposing intact mouse skeletal muscle fibers to NO donors increases [Ca\(^{2+}\)]\(_{\text{i}}\) during submaximal electrical stimulation. However, differences in experimental setup between the latter and the present study may partly explain these discrepancies. First, in between [Ca\(^{2+}\)]\(_{\text{i}}\) responses were studied during submaximal activation, whereas we investigated the effects of NO on maximal [Ca\(^{2+}\)]\(_{\text{i}}\) activation. Previous studies using an in situ skeletal muscle preparation indicated that the effects of NO depend on contraction pattern and frequency of stimulation (21). SNAP did not affect twitch force at 0.5 Hz but decreased twitch force at 1.5 and 4.0 Hz. Also, SNAP decreased tetanic force at 40 Hz but did not affect tetanic force at 12 Hz (21). Likewise, NO differently affects SR Ca\(^{2+}\) release in nonactivated and activated SR vesicles. In the former, NO donors increased Ca\(^{2+}\) release (26), whereas, in vesicles exposed to caffeine, NO donors decreased Ca\(^{2+}\) release (18). It is unknown how the degree of stimulation determines the effect of NO on force generation and Ca\(^{2+}\) release. Second, we determined the effect of NO donors on the amplitude of [Ca\(^{2+}\)]\(_{\text{i}}\) response, whereas Andrade et al. measured [Ca\(^{2+}\)]\(_{\text{omembr}}\) when a clear plateau was reached. As mentioned earlier, maximal amplitude and plateau of a [Ca\(^{2+}\)]\(_{\text{i}}\) response partly depend on different processes in Ca\(^{2+}\) handling. Indeed, Andrade et al. found that Ca\(^{2+}\) ATPase activity was decreased by S-nitroso-N-acetyl-cysteine, which could, at least partly, explain the observed elevation in [Ca\(^{2+}\)]\(_{\text{i}}\) during submaximal activation in their study. Together, the picture emerges that NO has complex effects on [Ca\(^{2+}\)]\(_{\text{i}}\) handling, acting on different steps in the excitation-contraction coupling cascade, and effects depend on concentration of NO and the extent of skeletal muscle activation.

In conclusion, Westerblad et al. (29) showed that [Ca\(^{2+}\)]\(_{\text{i}}\) in mouse skeletal muscle fibers was reduced during low-frequency fatigue, which was probably the result of impaired SR Ca\(^{2+}\) release. It has been shown that repetitive electrical stimulation of skeletal muscle at either 25 (12) or 100 Hz (4) enhances generation of NO. Our data indicate that NO impairs SR Ca\(^{2+}\) release. Thus it could be speculated that reduced SR Ca\(^{2+}\) release during low-frequency fatigue (partly) results from elevated generation of NO. However, further studies are needed to prove such a mechanism.

This study was financially supported by National Heart, Lung, and Blood Institute Grants HL-37680 and HL-34817 (G. C. Sieck), National Institute of General Medical Sciences Grant GM-57816 (Y. S. Prakash), and Dutch Asthma Foundation Grant 97.34 (L. M. A. Heunks).

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