Mechanism of Nitric Oxide–Induced Vasodilatation
Refilling of Intracellular Stores by Sarcoplasmic Reticulum Ca\(^{2+}\) ATPase and Inhibition of Store-Operated Ca\(^{2+}\) Influx

Richard A. Cohen, Robert M. Weisbrod, Marion Gericke, Mohammad Yaghoubi, Charlene Bierl, Victoria M. Bolotina

Abstract—The precise mechanisms by which nitric oxide (NO) decreases free [Ca\(^{2+}\)]\(_i\), inhibits Ca\(^{2+}\) influx, and relaxes vascular smooth muscle are poorly understood. In rabbit and mouse aorta, agonist-induced contractions and increases in [Ca\(^{2+}\)]\(_i\), were resistant to nifedipine, suggesting Ca\(^{2+}\) entry through non–L-type Ca\(^{2+}\) channels. Relaxations to NO were inhibited by thapsigargin (TG) or cyclopiazonic acid (CPA) indicating the involvement of sarcoplasmic reticulum ATPase (SERCA). Studies of the effect of NO on [Ca\(^{2+}\)]\(_i\), and the rate of Mn\(^{2+}\) influx with fura-2 fluorometry in rabbit aortic smooth muscle cells in primary culture were designed to test how SERCA is involved in mediating the response to NO. When cells were stimulated with angiotensin II (AII), NO accelerated the removal of Ca\(^{2+}\) from the cytoplasm, decreased [Ca\(^{2+}\)]\(_i\), and inhibited Ca\(^{2+}\) and Mn\(^{2+}\) influx. Inhibition of SERCA abolished all the effects of NO. In contrast, inhibition of the Na\(^+/Ca^{2+}\) exchanger or the plasma membrane Ca\(^{2+}\) ATPase had no influence on the ability of NO to decrease [Ca\(^{2+}\)]\(_i\), NO maximally decreased [Ca\(^{2+}\)]\(_i\) within 5 s, whereas significant inhibition of AII-induced Ca\(^{2+}\) and Mn\(^{2+}\) influx required more than 15 s. The inhibition of cation influx strictly depended on [Ca\(^{2+}\)]\(_i\), and functional SERCA, suggesting that during the delay before NO inhibits Ca\(^{2+}\) influx, the influx of Ca\(^{2+}\) and the uptake into intracellular stores are required. In the absence of [Ca\(^{2+}\)]\(_i\), NO diminished the AII-induced [Ca\(^{2+}\)]\(_i\), transient by a SERCA-dependent mechanism and increased the amount of Ca\(^{2+}\) in the stores subsequently released by ionomycin. The present study indicates that the initial rapid decrease in [Ca\(^{2+}\)]\(_i\) caused by NO in vascular smooth muscle is accounted for by the uptake of Ca\(^{2+}\) by SERCA into intracellular stores. It is proposed that the refilling of the stores inhibits store-operated Ca\(^{2+}\) influx through non–L-type Ca\(^{2+}\) conducting ion channels and that this maintains the decrease in [Ca\(^{2+}\)]\(_i\), and NO-induced relaxation. (Circ Res. 1999;84:210-219.)

Key Words: nitric oxide ■ Ca\(^{2+}\) ATPase ■ Ca\(^{2+}\) ■ Ca\(^{2+}\) stores ■ vascular smooth muscle

The endothelial cell production of nitric oxide (NO) accounts for endothelium-dependent vasodilatation. Despite a growing understanding of the mechanisms by which NO is released from the endothelium, the precise molecular mechanisms by which NO relaxes vascular smooth muscle are not fully understood. A variety of mechanisms have been proposed by which NO participates in the regulation of contractile tone. Among them, several different mechanisms for NO-induced inhibition of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels have been proposed, including their inhibition by cGMP-dependent mechanisms or by membrane hyperpolarization due to direct or indirect cGMP-dependent activation of K\(^+\) channels. NO also has been proposed to reduce [Ca\(^{2+}\)]\(_i\) by inhibiting the agonist-induced release of Ca\(^{2+}\) from intracellular stores or by increasing Ca\(^{2+}\) removal from the cytoplasm by accelerating the plasma membrane Ca\(^{2+}\) ATPase or the Na\(^+/Ca^{2+}\) exchanger. The sarcoplasmic(endo)plasmic reticulum Ca\(^{2+}\) ATPase (SERCA), which may be accelerated by the NO-induced rise in cGMP, is also thought to be involved. Thus, several potential Ca\(^{2+}\) regulatory targets have been proposed for NO in vascular smooth muscle, but their relative importance in decreasing [Ca\(^{2+}\)]\(_i\), and their individual contribution to smooth muscle relaxation is still unclear.

Supporting previous observations, the present study indicates that agonist-induced increases in [Ca\(^{2+}\)]\(_i\), and contractions of rabbit and mouse aortic smooth muscle cells are highly resistant to inhibitors of L-type Ca\(^{2+}\) channels, indicating that Ca\(^{2+}\) influx through different channels can mediate the responses. In a variety of cell types, including smooth muscle, Ca\(^{2+}\) entry is thought to be initiated by agonist-induced Ca\(^{2+}\) release and depletion of [Ca\(^{2+}\)]\(_i\) stores. Activation of store-operated Ca\(^{2+}\) entry is known to be triggered

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not only by receptor-dependent agonists but also by store depletion that occurs after inhibiting Ca\(^{2+}\) uptake into the stores by thapsigargin (TG) or cyclopiazonic acid (CPA).

Increases in [Ca\(^{2+}\)], and contractions stimulated by agonists including phenylephrine, angiotensin II (AII), or 5-hydroxytryptamine (5-HT) are shown in the present study to be highly sensitive to NO, but those caused by inhibitors of SERCA are demonstrated to be resistant to NO. This important finding led us to further investigate the effect of NO on [Ca\(^{2+}\)], stores and store-operated Ca\(^{2+}\) entry. Our results suggest a simple model whereby NO initiates its response by accelerating sequestration of Ca\(^{2+}\) into intracellular stores via SERCA, thereby rapidly decreasing [Ca\(^{2+}\)]. The resulting refilling of Ca\(^{2+}\) stores would indirectly inhibit store-operated Ca\(^{2+}\) influx, thereby maintaining the decrease in [Ca\(^{2+}\)], and mediating smooth muscle cell relaxation. This coordinated effect on [Ca\(^{2+}\)], homeostasis represents a novel mechanism for NO-induced vasodilatation.

**Materials and Methods**

**Relaxation of Intact Rabbit and Mouse Aorta**

Rings of New Zealand White rabbit descending thoracic aorta were cleaned of adherent fat and connective tissue and were suspended in organ chambers filled with physiological salt solution (PSS) for tension measurements as previously described. Rings (3 mm long) of the thoracic aorta of mice (8 to 11 weeks of age) were suspended on horizontal wire myographs (Kent Scientific). After equilibration at the optimal resting tension (mouse aorta, 1.5 g; rabbit aorta, 6 g), the smooth muscle was contracted with phenylephrine hydrochloride, and after achieving a stable contraction, NO was added in increasing concentrations. When needed, the contractile tone caused by SERCA inhibitors was supplemented with phenylephrine to increase the smooth muscle cell relaxation. This coordinated effect on [Ca\(^{2+}\)], homeostasis represents a novel mechanism for NO-induced vasodilatation.

**[Ca\(^{2+}\)]\(i\) Measurement in Intact Mouse Aorta**

The mouse aorta was slid over a Teflon filament to fix it and to destroy the endothelial layer. In a Petri dish containing ice-cold PSS (in mmol/L: NaCl 140, KCl 2.8, MgCl\(_2\) 1, CaCl\(_2\) 2, glucose 11, and HEPES 10 [pH 7.4]), the aorta was cleaned of fat and connective tissue and cut into 2-mm-long rings. Each ring was transferred into an experimental chamber (\(\Delta\)TC3 dish, Bioptechs, Inc) between 2 small plaques of neutral silicon grease. Here the ring was cut open, fixed by a piece of woven nylon mesh (Spectra/Mesh nylon, Spectrum) and stored in filtered PSS solution at 4°C until use, but not longer than 4 hours. Fura-2 loading of the strip was done in 10\(^{-3}\) mol/L fura-2 AM in PSS with 0.02% Pluronic F-127 (Molecular Probes) for 1 hour at 37°C. Strips were then washed in prewarmed PSS for 15 minutes at 37°C, and the chamber was mounted into a \(\Delta\)TC3 temperature control system (Bioptechs, Inc) on the stage of the microscope (Olympus, \(\times20\) fluorescence objective). The solution was warmed to 37°C. The microscope was focused on the elastin and collagen structure of the tissue beneath the nylon mesh. NO was applied as a 50-μL drop of 1 μmol/L solution close to the aortic strip. At the end of each measurement, digitorin and MnCl\(_2\) were applied in several steps to avoid contraction and dislocation of the strip, reaching final concentrations of 10\(^{-4}\) mol/L and 2 \(\times10^{-2}\) mol/L, respectively. Fluorescence background was automatically subtracted. Because of the well-known difficulties in calibrating fura-2 in intact tissues and obtaining precise values, [Ca\(^{2+}\)], changes are reported as ratios after background subtraction.

**Cell Culture**

The thoracic aorta was removed from New Zealand White rabbits and cleaned of adherent fat and connective tissue. Strips of rabbit thoracic aorta were aseptically removed from the media, dispersed with collagenase (4 mg/mL) and elastase (1 mg/mL), and grown in medium M199 with 20% heat-inactivated FBS as previously described. Cells were seeded onto 9×22 mm No. 1 glass coverslips for [Ca\(^{2+}\)], studies. On reaching confluence, cells were growth-arrested by placing them in M199 with 1% FBS for 3 to 4 days before the experiments. All experiments were done on primary cultures that stain uniformly for smooth muscle α-actin.

**[Ca\(^{2+}\)]\(i\) Measurement in Cultured Smooth Muscle**

As described previously, cells in culture on coverslips were loaded for 45 minutes with fura-2 AM (5 μmol/L) in PSS at 37°C and washed for 15 minutes to complete ester cleavage. The PSS was of the following composition (mmol/L): NaCl 119, NaHEPES 20, KCl 4.6, CaCl\(_2\) 1.2, MgSO\(_4\) 1.0, NaHPO\(_4\) 0.15, KH\(_2\)PO\(_4\) 0.4, and NaHCO\(_3\) 5.0, supplemented with 0.1% albumin. Nominally Ca\(^{2+}\)-free PSS was composed of the same solution without CaCl\(_2\) or EGTA added. Fura-2 fluorescence was measured in a photomultiplier-based fluorometer at 37°C or in an imaging system at 22°C to 24°C (Figure 3) (both from IonOptix Inc). Sampling rate for the ratio of 510-nm emitted light from excitation at 340 and 380 nm was 2 Hz (photomultiplier system) or 5 Hz (imaging system). [Ca\(^{2+}\)], in cultured cells was estimated by previously published methods, and the concentration is reported as nanomoles per liter (nmol/L).

**Measurement of Divalent Cation Influx Rate**

Mn\(^{2+}\)-induced quenching of fura-2 fluorescence was used to estimate rates of divalent cation influx. Fluorescence of fura-2–loaded cells was measured at the isosbestic point wavelength of 360 nm. Data are expressed as the Mn\(^{2+}\) influx rates, which were calculated from the slope of the linear decline in fura-2 fluorescence during the 30 s immediately after addition of MnCl\(_2\) (100 μmol/L), and were normalized to the level of fluorescence at the time of Mn\(^{2+}\) addition. When the rate of Mn\(^{2+}\) influx in the presence and absence of [Ca\(^{2+}\)], was compared, the rates were normalized to that measured under basal conditions.

**Measurement of Inositol 1,4,5-Trisphosphate**

Rabbit aortic smooth muscle cells were grown to confluence in 35-mm culture dishes and made quiescent by reducing the supplemental serum to 1% during the last 4 days before the experiment. Quiescent cells were incubated for 1 hour in serum-free PSS and then stimulated with 0.1 μmol/L AII for 15 s to 70 s. The extracellular solution was removed, and 250 μL of cold 1 mol/L trichloroacetic acid was added. The lysate was collected and centrifuged for 1 minute at 12,000g at 4°C. The trichloroacetic acid was extracted with a 3:1 solution of 1,1,2-trichloro-1,2,2-trifluoroethane-triethylamine, and the remaining inositol 1,4,5-trisphosphate (IP\(_3\)) was quantified according to the methodology provided with the IP\(_3\), \(^3\)H radioreceptor assay kit (Dupont NEN).

**Materials**

NO gas was obtained from Matheson. Saturated NO solution was prepared at 4°C and diluted in deoxygenated sealed tubes of aqueous solution as described. Responses of intact rings and cultured cells to NO (10\(^{-10}\) to 10\(^{-6}\) mol/L) were entirely prevented by deoxyhemoglobin (10 \(^{-3}\) mol/L). Fura-2 AM was from Molecular Probes. All other chemicals were from Sigma Chemical Co.

**Statistical Analysis**

All data are expressed as mean±SE. Evaluation of statistics was done by Student \(t\) test. Values of \(P<0.05\) indicated significant differences and are designated in the figures by an asterisk.

**Results**

**Effect of Nifedipine and Inhibitors of SERCA on NO-Induced Relaxations and Decreases in [Ca\(^{2+}\)], in Intact Rabbit and Mouse Aorta**

The role of L-type Ca\(^{2+}\) channels in agonist-induced contractions and NO-induced relaxations of rings of rabbit thoracic aorta...
denuded of endothelium was determined. Figure 1A (left) shows that phenylephrine (10⁻⁸ to 3 × 10⁻⁶ mol/L) caused concentration-dependent contractions, and NO (10⁻⁹ to 10⁻⁵ mol/L) caused concentration-dependent relaxations (Figure 1A, right). TG or CPA had no consistent direct effect on tone in resting aortic rings, and similar concentrations of phenylephrine were required to contract the rings to equivalent levels of tone. In the absence or presence of TG or CPA, respectively, the concentration of phenylephrine (−log mol/L: 7.2±0.5, 7.5±0.6, and 7.2±0.7) and the contraction produced (g: 7.5±1.1, 7.4±0.7, and 6.2±0.9) were not significantly different. There was a significant inhibition of the NO-induced relaxation caused by TG (P<0.05) or CPA (P<0.05), designated by asterisks.

The role of calcium uptake into intracellular stores in the relaxations to NO was determined in rabbit aortic rings pretreated with the SERCA inhibitors TG and CPA (Figure 1B). TG (10⁻⁴ mol/L) or CPA (2 × 10⁻⁴ mol/L) caused small, transient, and inconsistent contractions of resting rabbit thoracic aortic rings denuded of endothelium. The concentration of phenylephrine required to contract the rings in the presence of TG or CPA was unchanged (Figure 1B). Relaxations caused by NO (10⁻¹⁰ to 10⁻⁵ mol/L) were significantly inhibited by both TG and CPA (P<0.05, n=5; Figure 1B).
In rings of mouse aorta contracted with phenylephrine, NO caused rapid, transient, and concentration-dependent relaxations (Figure 2A). In the mouse aorta, TG consistently caused slowly developing contractions, whereas CPA caused small and variable contractions (Figure 2B and 2C). The tone induced in the presence of the inhibitors of SERCA, with or without supplemental phenylephrine, was not significantly affected by NO (Figure 2B through 2D).

To determine whether in the above experiments contraction and relaxation reflected changes in \([Ca^{2+}]_i\), intact mouse aortic strips were loaded with fura-2, and the effects of NO on agonist- and TG-induced increases in \([Ca^{2+}]_i\) were measured. 5-HT, used in these experiments because it was the most potent agonist, caused a rapid transient increase in \([Ca^{2+}]_i\), followed by a plateau (Figure 2E). NO significantly decreased the plateau level from a ratio of 0.045±0.011 to 0.014±0.010 \((P<0.02, n=9)\). Treatment of the mouse aorta with nifedipine 10 \(\mu\)mol/L did not significantly affect either the plateau (0.038±0.016) or the decrease caused by NO (0.016±0.009, \(n=6)\). The NO-induced decrease in \([Ca^{2+}]_i\), was 86±26% in the absence and 64±14% in the presence of nifedipine (\(P>0.1)\). TG caused a slow increase in \([Ca^{2+}]_i\), to a plateau value of 0.104±0.016, and NO did not significantly affect this response (0.099±0.013, \(P>0.1, n=4\); Figure 2F).

**SERCA Inhibitors Prevent the Decrease in \([Ca^{2+}]_i\) Caused by NO in Rabbit Aortic Smooth Muscle Cells in Culture**

The experiments discussed above showing that TG or CPA inhibited decreases in \([Ca^{2+}]_i\), and relaxations to NO in intact aortic tissue suggest that calcium uptake into intracellular stores by SERCA is involved in mediating the response to NO. Therefore, additional experiments of the effect of TG and CPA on \([Ca^{2+}]_i\), regulation by NO were performed in fura-2–loaded rabbit aortic smooth muscle cells in primary culture. In the presence of \([Ca^{2+}]_o\) (1.2 mmol/L), TG (100 nmol/L) caused a rise in \([Ca^{2+}]_i\), similar to that observed in intact mouse aorta. NO (10\(^{-6}\) mol/L) had no effect on the TG-induced increase in \([Ca^{2+}]_i\), when it was applied after \([Ca^{2+}]_i\), reached maximum level (Figure 3A). If NO was added 1 minute before TG, the rise in \([Ca^{2+}]_i\), caused by the SERCA inhibitor was slowed but reached a similar maximum level (Figure 3B). If NO was added 1 minute after addition of TG, it caused a small transient decrease in \([Ca^{2+}]_i\) (Figure 3C). Thus, in the presence of TG, there is a time-dependent disappearance of the response to NO.

**Figure 3.** TG causes a time-dependent inhibition of the response to NO. A, Increase in \([Ca^{2+}]_i\) caused by TG (100 nmol/L) in rabbit aortic smooth muscle cells in primary culture. After reaching the maximal response to TG, NO (1 \(\mu\)mol/L) had no effect. B, Addition of NO before TG slowed the increase in \([Ca^{2+}]_i\), but \([Ca^{2+}]_i\) eventually reached a similar level. C, NO added during the rise in \([Ca^{2+}]_i\) caused a transient decrease in \([Ca^{2+}]_i\). The 3 experiments shown were repeated 3 times with similar results.

**Figure 4.** Inhibiting SERCA, but not Na\(^+\)/Ca\(^{2+}\) exchange and PMCA, prevents the decrease in \([Ca^{2+}]_i\) caused by NO. All (A through C; 100 nmol/L) or TG (D and E; 100 nmol/L) was used to stimulate the release of \([Ca^{2+}]_i\); in the nominal absence of \([Ca^{2+}]_o\); as well as the influx of Ca\(^{2+}\), which occurred on the addition of \([Ca^{2+}]_o\); in rabbit aortic smooth muscle cells in primary culture. A, Elevation in \([Ca^{2+}]_i\); caused by All was insensitive to nifedipine (5 \(\mu\)mol/L) but was inhibited transiently by NO (10\(^{-10}\) and 10\(^{-7}\) mol/L) and blocked by Ni\(^{2+}\) (2 mmol/L). B, Under control conditions, NO (1 \(\mu\)mol/L) caused a sustained decrease in \([Ca^{2+}]_i\); C, Na\(^+\)/Ca\(^{2+}\) exchange was inhibited by replacing Na\(^+\) in the buffer with choline, and PMCA was inhibited by increasing Mg\(^{2+}\) in the buffer to 20 mmol/L and pH to 8.8. Under these conditions, the transient increase in \([Ca^{2+}]_i\); was unchanged, the increase in \([Ca^{2+}]_i\); that occurred on Ca\(^{2+}\) addition was increased, and the effect of NO was unchanged (see Table). When \([Ca^{2+}]_i\); was increased by adding Ca\(^{2+}\), after stimulation with TG (D; 100 nmol/L), NO had no effect, but addition of Ni\(^{2+}\) decreased \([Ca^{2+}]_i\);. When \([Ca^{2+}]_o\); was increased by TG to inhibit SERCA, and Na\(^+\)/Ca\(^{2+}\) exchange and PMCA (E) were also inhibited, \([Ca^{2+}]_i\); was significantly higher after addition of \([Ca^{2+}]_o\);, and neither NO nor Ni\(^{2+}\) decreased \([Ca^{2+}]_i\); (see Table).
Mechanism of NO-Induced Vasodilatation

Effect of Inhibiting [Ca\textsuperscript{2+}], Removal Mechanisms on the Decrease in [Ca\textsuperscript{2+}], Caused by NO and Ni\textsuperscript{2+} in Smooth Muscle Cells

<table>
<thead>
<tr>
<th>[Ca\textsuperscript{2+}], nmol/L (% Decrease)</th>
<th>Basal</th>
<th>Plateau</th>
<th>NO</th>
<th>Ni\textsuperscript{2+}</th>
</tr>
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<tbody>
<tr>
<td>All</td>
<td>39±5</td>
<td>251±13</td>
<td>114±7* (65±2%)*</td>
<td>74±7* (83±2%)*</td>
</tr>
<tr>
<td>(+ Inhibition of PMCA)</td>
<td>33±3</td>
<td>308±20†</td>
<td>103±8* (74±1%)*</td>
<td>67±6* (87±2%)*</td>
</tr>
<tr>
<td>(+ Inhibition of Na\textsuperscript{+}/Ca\textsuperscript{2+})</td>
<td>32±3</td>
<td>279±24</td>
<td>114±13* (67±3%)*</td>
<td>77±12* (84±3%)*</td>
</tr>
<tr>
<td>(+ Inhibition of both)</td>
<td>28±3</td>
<td>393±28†</td>
<td>130±12* (72±2%)*</td>
<td>81±8* (86±2%)*</td>
</tr>
<tr>
<td>TG (inhibition of SERCA)</td>
<td>36±4</td>
<td>409±13</td>
<td>403±16 (2±1%)</td>
<td>133±7* (74±2%)*</td>
</tr>
<tr>
<td>(+ Inhibition of PMCA and Na\textsuperscript{+}/Ca\textsuperscript{2+})</td>
<td>40±6</td>
<td>786±64†</td>
<td>781±60 (1±1%)</td>
<td>736±81 (8±4%)</td>
</tr>
<tr>
<td>CPA (inhibition of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger)</td>
<td>31±2</td>
<td>293±11</td>
<td>277±10 (6±1%)</td>
<td>64±4 (88±1%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE (n=5) of [Ca\textsuperscript{2+}] in nmol/L. Cells were placed in nominally Ca\textsuperscript{2+}-free solutions with additions to inhibit PMCA and/or Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, as described and shown in Figure 4. Ca\textsuperscript{2+} (1.2 mmol/L) was added 3 or 6 minutes after All (100 nmol/L), TG (100 nmol/L) or CPA (20 μmol/L), respectively; the plateau level [Ca\textsuperscript{2+}], was quantified when the level of [Ca\textsuperscript{2+}], stabilized. Minimum [Ca\textsuperscript{2+}], levels achieved after the addition of NO (1 μmol/L) or Ni\textsuperscript{2+} (2 mmol/L) are shown, along with the percentage of decrease from the plateau level of [Ca\textsuperscript{2+}], shown in parentheses.

*Significant decrease (P<0.05) in [Ca\textsuperscript{2+}], caused by NO or Ni\textsuperscript{2+}; †Significant increase (P<0.05) in the level of [Ca\textsuperscript{2+}], caused by inhibition of SERCA, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, and PMCA.

The effect of NO was studied further on All-induced increases in [Ca\textsuperscript{2+}]. In these experiments, All was used because, similar to phenylephrine and 5-HT, it activates IP\textsubscript{3}-induced Ca\textsuperscript{2+} release and influx, and it gave the most consistent response in the smooth muscle cells in culture. The All-induced elevation of [Ca\textsuperscript{2+}], was separated into the transient rise due to the release of Ca\textsuperscript{2+} from intracellular stores (shown to occur in the nominal absence of [Ca\textsuperscript{2+}]) and the sustained elevation in [Ca\textsuperscript{2+}], after the addition and influx of [Ca\textsuperscript{2+}], (Figure 4A). Similar to phenylephrine-induced contractions and 5-HT induced increases in [Ca\textsuperscript{2+}], in the intact rabbit and mouse aorta, the Ca\textsuperscript{2+} influx–induced sustained elevation of [Ca\textsuperscript{2+}], was insensitive to nifedipine (5 μmol/L) but was sensitive to NO (10\textsuperscript{-6} to 10\textsuperscript{-5} μmol/L), as well as to blocking Ca\textsuperscript{2+} influx by Ni\textsuperscript{2+} (Figure 4A).

NO (10\textsuperscript{-6} mol/L) applied during the elevation of [Ca\textsuperscript{2+}], caused by All-induced Ca\textsuperscript{2+} influx (Figure 4B) produced a very rapid and sustained decrease in [Ca\textsuperscript{2+}]. Because the response to NO could result not only from inhibition of Ca\textsuperscript{2+} influx but also from acceleration of Ca\textsuperscript{2+} extrusion from the cytoplasm via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and the plasma membrane Ca\textsuperscript{2+} ATPase (PMCA) or by sequestration of Ca\textsuperscript{2+} into the stores by SERCA, the effects of inhibition of these systems were determined. Inhibition of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (by replacing extracellular Na\textsuperscript{+} with choline) and/or PMCA (by elevating pH\textsubscript{i} to 8.8 and Mg\textsuperscript{2+} concentration to 20 mmol/L) increased the sustained elevation of [Ca\textsuperscript{2+}], but did not affect the NO-induced decrease in [Ca\textsuperscript{2+}], (Figure 4C; Table). In contrast, a similar Ca\textsuperscript{2+} elevation evoked by inhibition of SERCA with TG or CPA (Figure 4D; Table) was completely insensitive to NO (1 μmol/L). Blocking Ca\textsuperscript{2+} influx with Ni\textsuperscript{2+} decreased [Ca\textsuperscript{2+}], when either TG (Figure 1D) or PMCA and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (Figure 1C) were inhibited (Table). However, when SERCA, PMCA, and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger were all inhibited (Figure 1E), blocking Ca\textsuperscript{2+} influx with Ni\textsuperscript{2+} was not followed by a decrease in [Ca\textsuperscript{2+}]. This indicates that these 3 mechanisms are together responsible for eliminating Ca\textsuperscript{2+} from the cytoplasm and that they were effectively inhibited. These studies indicate that SERCA activity, but not that of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger or PMCA, is required for NO to decrease [Ca\textsuperscript{2+}].

NO-Induced Inhibition of Ca\textsuperscript{2+} and Mn\textsuperscript{2+} Influx Requires SERCA Activity and [Ca\textsuperscript{2+}]

After stimulation with All in the absence of [Ca\textsuperscript{2+}], treatment of cells with NO (1 μmol/L) significantly inhibited the rise in [Ca\textsuperscript{2+}], caused by the addition of [Ca\textsuperscript{2+}], (Figure 5A). The initial rise in [Ca\textsuperscript{2+}], started similarly in the absence or presence of NO, but in the presence of NO, [Ca\textsuperscript{2+}], stopped increasing and reached a stable plateau of 214±60 mmol/L after 21±4 s (n=3), compared with a peak of 560±89 mmol/L (P<0.05, n=3) reached in 18±2 s in the absence of NO. In contrast, NO did not affect the rise in [Ca\textsuperscript{2+}], caused by addition of Ca\textsuperscript{2+} in the presence of TG (Figure 5B; 551±20 and 531±28 mmol/L, n=3, with or without NO, respectively). These studies suggest that NO inhibits All- but not TG-induced Ca\textsuperscript{2+} influx.

To study cation influx while excluding the influence of mechanisms that remove Ca\textsuperscript{2+} from the cytoplasm, All-induced Mn\textsuperscript{2+} influx was measured. Figure 5C shows that NO increases the rate at which Mn\textsuperscript{2+} quenched fura-2 fluorescence. NO (1 μmol/L), applied 1 minute before Mn\textsuperscript{2+}, significantly inhibited cation influx caused by All. Interestingly, in the nominal absence of [Ca\textsuperscript{2+}], NO had no effect (Figure 5E). TG (100 nmol/L) increased Mn\textsuperscript{2+} influx to similar levels as All, but NO had no effect on Mn\textsuperscript{2+} influx in either the presence or absence of [Ca\textsuperscript{2+}], (Figure 5D and 5F).

Effect of NO on [Ca\textsuperscript{2+}] Precedes Its Effect on Cation Influx

Having investigated the effects of NO on cation influx, studies were conducted to determine the temporal relationship between the inhibition of cation influx and the
Figure 5. NO inhibits the influx of [Ca\(^{2+}\)]\(_i\) and Mn\(^{2+}\) caused by AII but not by TG. [Ca\(^{2+}\)]\(_i\) was measured in rabbit aortic smooth muscle cells in primary culture. A, When administered after the transient response to AII (100 nmol/L) in the absence of [Ca\(^{2+}\)]\(_o\), NO (10\(^{-5}\) mol/L) inhibited the increase in [Ca\(^{2+}\)]\(_i\), associated with the subsequent addition of [Ca\(^{2+}\)]\(_o\). B, When administered after the response to TG (100 nmol/L) in the absence of [Ca\(^{2+}\)]\(_o\), NO had no observable effect on the increase in [Ca\(^{2+}\)]\(_i\), associated with the addition of [Ca\(^{2+}\)]\(_o\). C and D, Traces show quenching of 360-nm fura-2 fluorescence by Mn\(^{2+}\). Dotted lines indicate the initial slope that reflects the rate of Mn\(^{2+}\) influx over the initial 30 s after addition of Mn\(^{2+}\). E (100 nmol/L) or TG (D, 100 nmol/L) caused similar increases in the rate of Mn\(^{2+}\) quenching compared with the basal rate assessed without the addition of an agonist. When NO (1 \(\mu\)mol/L) was added 1 minute before Mn\(^{2+}\) in cells stimulated by AII, but not in those stimulated by TG, the influx of Mn\(^{2+}\) was inhibited. E and F, Summary of rates of Mn\(^{2+}\) influx, normalized to the rate under basal conditions. In the presence (hatched bars), but not in the absence of [Ca\(^{2+}\)]\(_o\) (solid bars), NO significantly decreased AII-stimulated Mn\(^{2+}\) influx. Mn\(^{2+}\) influx stimulated by TG was unaffected by NO in the presence or absence of [Ca\(^{2+}\)]\(_o\). Bars represent mean±SEM of results from 3 experiments.

Figure 6. NO decreases [Ca\(^{2+}\)]\(_i\) faster than Ni\(^{2+}\) but inhibits Mn\(^{2+}\) influx slower than Ni\(^{2+}\). The effect of NO (A and C) is compared with that of blocking cation channels directly with Ni\(^{2+}\) (B and D; 2 mmol/L) on [Ca\(^{2+}\)]\(_i\). A and B, Mn\(^{2+}\) influx (C and D) stimulated by AII (100 nmol/L) in rabbit aortic smooth muscle cells in primary culture. A, NO (1 \(\mu\)mol/L) decreases [Ca\(^{2+}\)]\(_i\) faster (half-time, \(\tau\)=1.4±0.1 s) than does Ni\(^{2+}\) (B; \(\tau\)=3.1±0.2 s). Example traces are from 4 similar experiments. C and D, Effect of NO (1 \(\mu\)mol/L) or Ni\(^{2+}\) (2 mmol/L) on the AII-stimulated influx of Mn\(^{2+}\). No or Ni\(^{2+}\) was added simultaneously with (0 s) or 15 s and 60 s before adding Mn\(^{2+}\). Unlike Ni\(^{2+}\), which inhibited Mn\(^{2+}\) influx when added at any time before adding Mn\(^{2+}\), NO had no effect on Mn\(^{2+}\) influx when added simultaneously with Mn\(^{2+}\). It was not until after 15 s that a significant inhibition of Mn\(^{2+}\) influx by NO was noted (*\(P<0.05\)). Bars represent mean±SEM of 3 experiments.

All-induced Mn\(^{2+}\) influx (Figure 6D), consistent with its blocking cation-conducting channels directly. In contrast, when NO was added simultaneously with Mn\(^{2+}\), the cation influx (estimated over the next 30 s) was not significantly different from that obtained with AII alone (Figure 6C). Only if NO was added >15 s before the addition of Mn\(^{2+}\) was there sufficient time for NO to inhibit AII-induced Mn\(^{2+}\) influx. This delay in the action of NO is similar to that needed for inhibition of Ca\(^{2+}\) influx, observed in Figure 5A, and suggests why after addition of Mn\(^{2+}\), [Ca\(^{2+}\)]\(_o\) begins to rise normally, despite treatment with NO. These results indicate that a considerable delay occurs before NO inhibits the influx of extracellular cations and that the decrease in [Ca\(^{2+}\)]\(_i\), caused by NO occurs more rapidly than can be accounted for by a direct inhibition of channel proteins. This finding is consistent with the initial rapid decrease in [Ca\(^{2+}\)]\(_i\), by NO being primarily due to stimulation of [Ca\(^{2+}\)] removal mechanisms.

NO Accelerates the Removal of Ca\(^{2+}\) From the Cytoplasm via SERCA and Promotes Refilling of [Ca\(^{2+}\)]\(_o\) Stores

Because the intracellular action of NO precedes its effect on Ca\(^{2+}\) influx, the effect of NO on the rate of removal of
Ca\textsuperscript{2+} from the cytoplasm was further studied. When AII-induced Ca\textsuperscript{2+} influx was stopped by rapidly eliminating Ca\textsuperscript{2+} from the extracellular solution, [Ca\textsuperscript{2+}], decreased with a half-time for exponential decay, \( \tau \), of 21±4.6 s (n=5; Figure 7A, left). The NO donor S-nitroso-N-acetylpenicillamine (SNAP, 10\textsuperscript{-4} mol/L) added at the same time that [Ca\textsuperscript{2+}], was eliminated significantly increased the rate at which [Ca\textsuperscript{2+}], declined, indicating that NO accelerates the removal of Ca\textsuperscript{2+} from the cytoplasm (\( \tau=8.1\pm1.1 \) s, n=5, \( P<0.04; \) Figure 7A, right). When Ca\textsuperscript{2+} was readmitted to the extracellular solution in the presence of SNAP, [Ca\textsuperscript{2+}], initially rose rapidly and then after a delay of 10 s to 15 s, reached a new plateau that was lower than in the absence of SNAP (Figure 7A, right).

Ca\textsuperscript{2+} influx stimulated by TG elevated [Ca\textsuperscript{2+}], to a level similar to that caused by AII. Elimination of [Ca\textsuperscript{2+}], resulted in a decline in [Ca\textsuperscript{2+}], at a rate that was not significantly different from that observed in the presence of AII (\( \tau=27\pm1.5 \) s, n=4, \( P>0.3; \) Figure 7B, left). This suggests that in the absence of Ca\textsuperscript{2+} influx, [Ca\textsuperscript{2+}], removal mechanisms other than SERCA are primarily responsible for decreasing [Ca\textsuperscript{2+}], in the presence of either AII or TG. However, inhibition of SERCA with TG completely prevented the increase in the rate of [Ca\textsuperscript{2+}], removal caused by SNAP (\( \tau=27\pm2.6 \) s, \( P>0.7; \) Figure 7B, right). This result supports the concept that NO rapidly decreases [Ca\textsuperscript{2+}], in the presence of AII primarily by accelerating the removal of cytosolic Ca\textsuperscript{2+} via SERCA.

To determine if NO accelerates the removal of cytoplasmic Ca\textsuperscript{2+} by increasing its uptake into intracellular stores, the effect of NO was determined on the Ca\textsuperscript{2+} content of the stores. Ionomycin (10 \mu mol/L) alone or in combination with AII (0.1 \mu mol/L) was used to release Ca\textsuperscript{2+} from the stores in the nominal absence of [Ca\textsuperscript{2+}]. Ionomycin alone caused a peak increase in [Ca\textsuperscript{2+}], from a basal value of 92±9 to 835±72 nmol/L (Figure 8A). Application of NO (1 \mu mol/L) 4 minutes before had no significant effect on the subsequent response to ionomycin (\( \sim7\pm9\% \) compared to without NO, not shown in figure). AII (0.1 \mu mol/L) caused a peak transient increase in [Ca\textsuperscript{2+}], to 591±33 nmol/L and significantly reduced the release caused by the subsequent application of ionomycin (442±22 nmol/L, n=6, \( P<0.003; \) Figure 8A). When NO (1 \mu mol/L) was given 1 minute before AII, the [Ca\textsuperscript{2+}], transient caused by AII was significantly reduced (434±40 nmol/L, \( P<0.007 \)), whereas the subsequent ionomycin-induced release was increased by 42±7\% compared with that in the absence of NO (631±47 nmol/L, \( P<0.003; \) Figure 8B). Even if NO was added 20 s after AII (after the peak increase in [Ca\textsuperscript{2+}],...
caused by the agonist), the ionomycin-induced release was 26±9% greater than in the absence of NO (564±63, P<0.04; Figure 8C), consistent with an NO-induced augmentation of the refilling of Ca\(^{2+}\) stores.

Inhibition of the peak increase in [Ca\(^{2+}\)], caused by AII could be achieved by either inhibition of Ca\(^{2+}\) release from the stores\(^{7,19}\) or by accelerated sequestration of Ca\(^{2+}\) into the stores. The role of uptake by SERCA was determined by releasing Ca\(^{2+}\) from the stores with AII in the absence or presence of TG. In the absence of [Ca\(^{2+}\)]\(_{i}\), NO (1 μmol/L), added before AII, inhibited the transient increase in [Ca\(^{2+}\)], caused by AII by 44±7% (Figure 9A and 9B). The peak increase in [Ca\(^{2+}\)], was 528±57 nmol/L compared with 319±52 nmol/L in the presence of NO. Two minutes after treatment with TG, AII caused a transient Ca\(^{2+}\) increase to a level that was not significantly different from that in the absence of TG (Figure 9C and 9D). However, NO failed to inhibit the AII response when SERCA was inhibited by TG (Figure 9C and 9D). Similarly, NO inhibited the Ca\(^{2+}\) influx-induced rise in [Ca\(^{2+}\)], caused by releasing the stores with AII but not with AII and TG. This suggests that NO inhibits the peak increase in [Ca\(^{2+}\)], caused by AII primarily by accelerating the sequestration of Ca\(^{2+}\) into the stores via SERCA, rather than by interfering with IP\(_3\)-induced Ca\(^{2+}\) release.

**NO Does Not Affect IP\(_3\) Production Caused by AII**

The effect of NO on AII-induced IP\(_3\) production was measured by radioimmunoassay. Basal levels of IP\(_3\) were 0.9±0.1 pmol/plate. During the first 45 s after AII (0.1 μmol/L), IP\(_3\) levels were significantly increased (3.1±0.5, 3.1±0.7, and 3.1±0.5 pmol/plate at 15, 30, and 45 s after AII, respectively, n=5) but were not significantly different after 1 minute of treatment with NO (3.4±0.6, 2.7±0.4, 2.2±0.6 pmol/plate, respectively). Furthermore, IP\(_3\) levels 60 s and 70 s after AII were 2.3±0.4 and 2.1±0.3 pmol/plate, respectively. When NO was administered at 60 s after AII, the level of IP\(_3\) 10 s later was not significantly decreased compared with that in the absence of NO (2.0±0.4 pmol/plate). Thus, changes in AII-induced IP\(_3\) levels do not explain the effect of NO on [Ca\(^{2+}\)].

**Discussion**

According to the capacitative Ca\(^{2+}\) entry model, Ca\(^{2+}\) influx can be activated not only by emptying the stores with agonists but also by depletion of the stores after inhibition of SERCA with TG or CPA.\(^{13-15}\) An important role of SERCA in the physiological response to NO in agonist-stimulated smooth muscle cells is suggested by the present study in which these 2 structurally different, highly specific inhibitors of SERCA\(^{20,21}\) interfered with the actions of NO. The present study also demonstrates that the initial action of NO in agonist-stimulated smooth muscle cells is to rapidly sequester [Ca\(^{2+}\)], via SERCA and secondarily to inhibit Ca\(^{2+}\) influx. This temporal sequence suggests the proposal that NO regulates agonist-induced Ca\(^{2+}\) influx by a 2-step process: (1) NO initiates a rapid decrease in [Ca\(^{2+}\)], by sequestering cytoplasmic Ca\(^{2+}\) into intracellular stores via SERCA, and (2) the refilling of intracellular stores inhibits store-operated Ca\(^{2+}\) entry, which in turn maintains the decrease in [Ca\(^{2+}\)], and relaxation of vascular smooth muscle.

Several pieces of evidence support sequestration of [Ca\(^{2+}\)], by SERCA as the primary mechanism by which NO initiates the decrease in [Ca\(^{2+}\)]. First, NO causes [Ca\(^{2+}\)], to fall more rapidly than can be explained by either directly inhibiting Ca\(^{2+}\) influx with Ni\(^{2+}\) (Figure 6) or by removing [Ca\(^{2+}\)]\(_{i}\). (Figure 7). Second, NO does not decrease [Ca\(^{2+}\)], when SERCA is inhibited by TG or CPA (Figures 2 through 4; Table). The ability of the inhibitors of SERCA to prevent the response to NO is time-dependent (Figure 3), presumably indicating that the inhibitors require sufficient time to completely block uptake of Ca\(^{2+}\) into the stores. Third, studies in which PMCA or Na\(^{+}/\)Ca\(^{2+}\) exchanger was inhibited (Figure 4) indicate that although these mechanisms actively remove [Ca\(^{2+}\)], from the cytoplasm when it is elevated by either AII or TG, they are not essential for NO to decrease [Ca\(^{2+}\)].

Figure 9. Inhibition of SERCA prevents the effect of NO on the response to AII in the absence of [Ca\(^{2+}\)]. The [Ca\(^{2+}\)]\(_{i}\) response to AII (100 nmol/L) was measured in rabbit aortic smooth muscle cells in primary culture in the nominal absence of [Ca\(^{2+}\)], and then after the addition of Ca\(^{2+}\); (A; 1.2 mmol/L). A, Traces are overlapped to show that when administered 3.6 minutes before AII, NO (+NO, 1 μmol/L) significantly decreased the peak transient rise in [Ca\(^{2+}\)], caused by AII, as well as the increase in [Ca\(^{2+}\)]\(_{i}\) after the addition of Ca\(^{2+}\); compared with the absence of NO (–NO; B). In cells first treated with TG (C and D; 100 nmol/L, 2.6 minutes before AII), AII still released [Ca\(^{2+}\)],. However, NO added before TG and AII had no significant effect on the AII-induced rise in [Ca\(^{2+}\)]\(_{i}\) nor in the rise in [Ca\(^{2+}\)]\(_{o}\) that followed the addition of 1.2 mmol/L [Ca\(^{2+}\)].
The ability of NO to increase the content of \([\text{Ca}^{2+}]_i\), stores was made evident by the increased size of the response to ionomycin after NO was administered before the partial emptying of the stores caused by AII (Figure 8). Ionomycin, at the concentrations used, is known to permeabilize membranes to \(\text{Ca}^{2+}\); to release all \(\text{Ca}^{2+}\) from stores, and to make \(\text{Ca}^{2+}\) pumps and transporters ineffective.22 In the absence of \([\text{Ca}^{2+}]_i\), an increased amount of \(\text{Ca}^{2+}\) released by ionomycin in the presence of NO can only be explained by increased uptake from the cytoplasm or decreased release from the stores. NO increased the ionomycin-releasable store even when NO was administered after \(\text{Ca}^{2+}\) release induced by AII was nearly complete, suggesting that NO increased reuptake, rather than inhibited the IP\(_3\)-mediated release of \(\text{Ca}^{2+}\) from the stores. Indeed, the effect of NO on the AII-induced transient increase in \([\text{Ca}^{2+}]_i\), in the absence of \([\text{Ca}^{2+}]_i\), was entirely prevented by pretreatment with TG (Figure 9). The latter experiment suggests that NO has little or no direct effect on the production of, or response to, IP\(_3\) initiated by all in these cells. This finding is also supported by the lack of an effect of NO on AII-stimulated IP\(_3\) levels. Thus, our results indicate that NO initiates a decrease in \([\text{Ca}^{2+}]_i\), primarily as a result of its sequestration into intracellular stores by SERCA.

The hypothesis that the inhibition of \(\text{Ca}^{2+}\) influx by NO is secondary to its initial effects on sequestration of \(\text{Ca}^{2+}\), into the stores by SERCA is based on several observations. The inhibition by NO of AII-induced Mn\(^{2+}\) influx was strictly dependent on \([\text{Ca}^{2+}]_i\), (Figure 5). This result suggests the apparent paradox that \(\text{Ca}^{2+}\) influx is required for NO to inhibit divalent cation influx. Indeed, when \([\text{Ca}^{2+}]_i\), was added after AII in the presence of NO (Figures 5, 7, and 9), \([\text{Ca}^{2+}]_i\), initially rose rapidly but then equilibrated at a lower level compared with control. This requirement for \(\text{Ca}^{2+}\) influx for NO to inhibit \(\text{Ca}^{2+}\) influx is also evident from the delay observed in the NO-induced inhibition of Mn\(^{2+}\) influx (Figure 6). Together with the lack of effects of NO when SERCA is inhibited, these experiments support a requirement for SERCA-dependent uptake of \(\text{Ca}^{2+}\) into the stores for divalent cation influx to be inhibited by NO.

Thus, our results can best be explained by proposing that NO indirectly inhibits capacitative or store-operated, agonist-activated \(\text{Ca}^{2+}\) influx in vascular smooth muscle cells by increasing \(\text{Ca}^{2+}\) in the stores. The observation that inhibitors of SERCA can prevent the actions of NO on \([\text{Ca}^{2+}]_i\), was made previously in studies on platelets23 and HEK 293 cells,24 but the finding was interpreted as meaning that NO is not capable of regulating capacitative \(\text{Ca}^{2+}\) influx. However, the present findings suggest that functional SERCA is an essential requirement for NO to regulate store-operated \(\text{Ca}^{2+}\) influx.

Although we have not measured a direct effect of NO on SERCA itself, others have shown that nitrvasodilators stimulate the ATPase activity of SERCA by a mechanism dependent on cGMP and protein kinase G.10 Furthermore, cGMP-dependent phosphorylation of phospholamban is induced by NO in vascular smooth muscle.10,25 Dissociation of phosphorylated phospholamban from SERCA would provide a molecular mechanism for NO to regulate SERCA activity and thus to regulate store-operated \(\text{Ca}^{2+}\) influx. cGMP-independent effects of NO on SERCA via redox-sensitive thiols are potentially possible, similar to those described recently for \(K^+\) channels8 and the ryanodine receptor.26

Indeed, the effects of NO on vascular smooth muscle \([\text{Ca}^{2+}]_i\), and relaxation persist despite effective inhibition of cGMP and protein kinase G.27

\(\text{Ca}^{2+}\) entry in vascular smooth muscle is known to occur via 2 distinct pathways, only one of which is sensitive to inhibitors of L-type \(\text{Ca}^{2+}\) channels.12 In the present study, nifedipine had little effect on agonist-induced increases in \([\text{Ca}^{2+}]_i\), and contractions of rabbit or mouse aortic smooth muscle, indicating that non-L-type \(\text{Ca}^{2+}\) channels are responsible for the majority of \(\text{Ca}^{2+}\) entry in these conduit arteries. Thus, the mechanism proposed in the present study can explain how NO regulates the portion of \(\text{Ca}^{2+}\) influx and contractions induced by agonists and growth factors that occurs independently of L-type \(\text{Ca}^{2+}\) channels. The voltage-dependent activation of smooth muscle L-type \(\text{Ca}^{2+}\) channels also can be regulated by NO either directly3 or indirectly by hyperpolarization mediated by \(K^+\) channels.4–6 Thus, the proposal that NO regulates agonist-activated, store-operated \(\text{Ca}^{2+}\) influx widens the potential mechanisms by which NO causes vasodilatation.

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References


Mechanism of Nitric Oxide–Induced Vasodilatation: Refilling of Intracellular Stores by Sarcoplasmic Reticulum Ca2+ ATPase and Inhibition of Store-Operated Ca2+ Influx

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