Nitric Oxide Inhibits Capacitative Cation Influx in Human Platelets by Promoting Sarcoplasmic/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase–Dependent Refilling of Ca\(^{2+}\) Stores

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Abstract—Nitric oxide (NO) is a potent inhibitor of thrombin-induced increase in cytoplasmic free Ca\(^{2+}\) concentration and aggregation in platelets, but the precise mechanism of this inhibition is unclear. To measure Ca\(^{2+}\)/Mn\(^{2+}\) influx in intact platelets and to monitor Ca\(^{2+}\) uptake into the stores in permeabilized platelets, fura-2 was used. In intact platelets, maximal capacitative Ca\(^{2+}\) and Mn\(^{2+}\) influx developed rapidly (within 30 s) after fast release of Ca\(^{2+}\) from the stores with thrombin (0.5 U/mL) or slowly (within 5 to 10 minutes) following passive Ca\(^{2+}\) leak caused by inhibition of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) with 30 \(\mu\)mol/L 2,5-di-(tert-butyl)-1,4-benzohydroquinone (BHQ). NO (1 \(\mu\)mol/L) inhibited capacitative Ca\(^{2+}\) and Mn\(^{2+}\) influx independently of the time after thrombin application. In contrast, the effect of NO on BHQ-induced Ca\(^{2+}\) and Mn\(^{2+}\) influx was observed only during the first few minutes after BHQ application and completely disappeared when capacitative cation influx reached its maximum. In Ca\(^{2+}\)-free medium, NO reduced the peak Ca\(^{2+}\) rise caused by thrombin and significantly promoted Ca\(^{2+}\) back-sequestration into the stores. Both effects disappeared in the presence of BHQ. Inhibition of guanylate cyclase with H-(1,2,4) oxadiazolo(4,3-a) quinoxallin-1-one (10 \(\mu\)mol/L) attenuated but did not prevent the effects of NO on cytoplasmic free Ca\(^{2+}\) concentration. Inhibition of Ca\(^{2+}\) uptake by mitochondria did not change the effects of NO. In permeabilized platelets, NO accelerated back-sequestration of Ca\(^{2+}\) into the stores after inositol-1,4,5-trisphosphate–induced Ca\(^{2+}\) release or after addition of Ca\(^{2+}\) (1 \(\mu\)mol/L) in the absence of inositol-1,4,5-trisphosphate. The effect of NO depended on the initial rate of Ca\(^{2+}\) uptake and on the concentration of ATP and was abolished by BHQ, indicating the direct involvement of SERCA. These data strongly support the hypothesis that NO inhibits store-operated cation influx in human platelets indirectly via acceleration of SERCA-dependent refilling of Ca\(^{2+}\) stores. (Circ Res. 1999;84:201-209.)

Key Words: nitric oxide ■ sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase ■ cation influx ■ Ca\(^{2+}\) ■ platelets

A n elevation in cytoplasmic free Ca\(^{2+}\) concentration (Ca\(^{2+}\)\(_{cyt}\)) is a major component of the signal transduction following receptor stimulation by thrombin in platelets.\(^{1}\) Thrombin activates phospholipase C, which generates inositol-1,4,5-trisphosphate (IP\(_3\)) and leads to the rapid depletion of IP\(_3\)-sensitive Ca\(^{2+}\) stores\(^{2,3}\) and initiation of cation influx.\(^{4-6}\) Similar to other types of nonexcitable cells,\(^{7}\) agonist-activated Ca\(^{2+}\) influx in platelets is thought to be capacitative in nature, being also activated by passive store depletion with an inhibitor(s) of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), thapsigargin (TG) or 2,5-di-(tert-butyl)-1,4-benzohydroquinone (BHQ).\(^{8-11}\)

Nitric oxide (NO), a potent inhibitor of thrombin-activated platelet aggregation,\(^{12,13}\) is known to decrease Ca\(^{2+}\)\(_{esc}\) that is raised by agonists,\(^{14}\) although the mechanism is not well defined. It has been shown that NO inhibits thrombin- and IP\(_3\)-stimulated Ca\(^{2+}\) release from internal stores in intact\(^{15}\) and permeabilized platelets.\(^{16,17}\) However, it is still unclear whether NO also inhibits capacitative cation influx. Brune et al\(^{17}\) found a significant inhibitory effect of the NO donor, sodium nitroprusside, on Mn\(^{2+}\) influx in TG-treated platelets. In contrast, Okamoto et al\(^{18}\) did not find an effect of the NO donor on TG-induced Ca\(^{2+}\) influx in human platelets and concluded that capacitative Ca\(^{2+}\) influx is resistant to NO. The data presented here demonstrate that NO, indeed, is a potent inhibitor of capacitative cation influx in human platelets and that SERCA activity is required for this effect. This finding is consistent with the idea that NO inhibits capacitative cation influx indirectly by promoting SERCA-dependent refilling of intracellular Ca\(^{2+}\) stores.

Materials and Methods

Platelet Isolation Procedure

Blood from healthy adult volunteers was drawn into plastic nonsterile tubes containing 10% volume of anticoagulant. The composition of anticoagulant was (in g/100 mL): trisodium citrate 2.5, citric acid
1.5, and glucose 2.0. To obtain platelet-rich plasma (PRP), the anticoagulated blood was centrifuged for 10 minutes at 350g at room temperature. Aspirin (100 μmol/L) was added to PRP to inhibit the activation of the platelets by thromboxane A2. Platelets were used within 5 hours after preparation.

For experiments on permeabilized platelets, cells were obtained by gel filtration as follows. Prewashed Sepharose-2B gel (Sigma) was placed into the plastic column with the 70-μm nylon mesh attached to the bottom and equilibrated with a 3X volume of standard HEPES buffer of the following composition (in mmol/L): NaCl 137, KCl 3.3, glucose 5.5, and HEPES 3.8 (pH 7.4). Ca2+ was not added to the buffer, but its residual concentration was 1 to 3 μmol/L as measured by Ca2+-sensitive electrode. Buffer was supplemented with 100 μmol/L aspirin and 6 μM apyrase to prevent platelet activation with thromboxane A2, and traces of ADP, respectively. After collection, the platelet suspension was stored at 37°C and used during the next 3 to 4 hours. The cell number in the suspension used for experiments was 2 to 2.5×10^9/mL.

Fluorescence Measurements
The fluorescent probe fura-2/AM was used to monitor changes in Ca2+ in platelets. PRP was diluted with standard buffer to the final cell number of ~1×10^8/mL and centrifuged at 750g for 10 minutes. Platelets were resuspended in the same buffer containing 2.5 μmol/L fura-2/AM and incubated at 37°C for 10 minutes. The platelet suspension was then centrifuged at 750g for 10 minutes and resuspended in 2 mL of buffer immediately before the fluorescence measurements. Cells were loaded with fura-2 before each run. Each experiment was done with 6 to 8×10^7 cells/mL. Fluorescence (F) measurements were carried out at 37°C using a spectrofluorimeter (Hitachi F-4500) with excitation wavelength alternating between 340 and 380 nm every 0.5 s and emission wavelength 510 nm. Changes in Ca2+ were estimated from the F340/F380 ratio (R340/380). Data collection, ratio calculation, and analysis were performed with the Hitachi software. All the recordings were corrected for the cell autofluorescence determined in unloaded platelets. In some experiments the initial rate of fall in Ca2+ concentration caused by NO in thrombin-stimulated platelets, and after addition of IP3, or Ca2+ in permeabilized platelets, was estimated from the initial slope during the first 5 to 20 seconds. Importantly, control experiments showed no evidence for a direct effect of NO either on response of fura-2 to Ca2+ or on the fluorescence properties of fura-2. To study cation influx, Mn2+ (100 to 300 μmol/L) was added to the platelets resuspended in standard buffer, and the rate of fura-2 quenching at an excitation and emission wavelength of 360 and 510 nm, respectively, was determined. The rate of Mn2+ influx was estimated from the slope during the first 60 s after Mn2+ addition, and the linear fit was performed with Microcal Origin software.

Experiments on Permeabilized Cells
Before the experiment, platelets were resuspended in an artificial cytoplasmic solution containing (in mmol/L): KCl 110, NaCl 10, KH2PO4 1, KHCO3 5, and HEPES 20 (pH 7.1) and supplemented with 1 μg/mL oligomycin, 2 μg/mL antimycin, 2 mmol/L MgATP, and an ATP-regenerating system containing 5 mmol/L creatine phosphate and 15 μmol/L creatine kinase. There was no Ca2+ added, and its residual concentration in the cytoplasmic solution was 1 to 3 μmol/L (as measured by a Ca2+-sensitive electrode). Fura-2 (K+ salt, 1 μmol/L) was added to this solution to monitor the changes in free Ca2+. During the experiment 20 μg/mL saponin was applied directly to the recording chamber, causing permeabilization of the plasmalemma membrane and resulting in sequestration of external Ca2+ into the stores, lowering the free Ca2+ concentration (see Figure 7A). After 4 to 6 minutes, Ca2+ concentration stabilized at a level of 200 to 250 nmol/L as calculated from the ratio F340/F380 assuming a Ks for fura-2 of 224 nmol/L.

NO Solution
A standard 1-L intravenous bag was filled with distilled water (750 mL) that had been bubbled with nitrogen gas to remove oxygen. Approximately 30 mEq of Bio-Rad analytical-grade anion exchange resin was mixed in the water before the bag was filled. The resin retains any nitrites or nitrates that may be formed by the reaction of NO with oxygen. The contents of the bag were bubbled with nitrogen gas for another 30 minutes. The contents were then mixed thoroughly, and the bag was placed in a refrigerator at 4°C. The concentration of NO in the solution equilibrated to give a 3.1±0.6 mmol/L (n=5) saturated solution that was stable at least for 1 week as measured by a chemiluminescent nitric oxide analyzer (Sievers NOA model 270). The absence of contaminating nitrite in saturated NO solution was confirmed by obtaining a similar analysis in the presence and absence of a reducing agent in the reflux chamber of the analyzer (KI in glacial acetic acid). At the time of the experiment, subsequent dilutions were made from the saturated NO solution by drawing off the solution from the bag with a gas-tight syringe and dissolving it in sealed tubes filled with deoxygenated solution (bubbled with nitrogen for 1 hour).

Materials
NO gas was from Matheson. Fura-2/AM and fura-2 (K+ salt) were from Molecular Probes. BHQ and IP3 were from Calbiochem. All other drugs were from Sigma.

Statistical Analysis
Each experiment was repeated 3 to 9 times. ANOVA and paired t test were used to determine the statistical significance of differences in obtained data. P<0.05 was considered significant. The results on the bar graphs are expressed as mean±SE.

Results
Cation Influx in Human Platelets Depends on Ca2+ Store Emptying
Although the existence of capacitative cation influx in platelets is widely accepted, the only strong evidence for it is the fact that TG and BHQ, inhibitors of SERCA that have been shown to deplete Ca2+ stores without elevation of IP3,20 cause a sustained Ca2+ influx8–11 similar to that activated by thrombin.4–6 To study the effect of NO on capacitative cation influx, the time-dependent relationship between Ca2+ store emptying and cation influx was first determined. For this purpose, cation influx after the rapid store emptying caused by thrombin was compared with the influx induced by the slower store emptying caused by inhibition of back-sequestration into the stores by BHQ.

Thrombin-Induced Cation Influx
To experimentally separate intracellular Ca2+ release from cation influx from the extracellular space, thrombin was applied at a submaximal concentration (0.5 U/mL) to suspensions of fura-2-loaded platelets in Ca2+-free medium. Cation influx was then analyzed following the addition of Ca2+ (1 mmol/L) or Mn2+ (100 μmol/L). A typical experiment (Figure 1A and 1B) shows that application of thrombin in the absence of extracellular Ca2+ caused a very rapid increase in Ca2+ that, after reaching a maximum, declined to a lower steady-state level. Adding extracellular Ca2+ after thrombin caused an immediate further increase in Ca2+ resulting from Ca2+ influx (Figure 1A and 1B). The amplitude of the maximal Ca2+ rise, determined mainly by Ca2+ influx, depended on the period between the addition of thrombin and Ca2+. Figure 2A summarizes the time dependence of thrombin-induced Ca2+ influx, showing that it was maximal when Ca2+ was added 30 s after thrombin and that it
decreased as the period between addition of thrombin and Ca\(^{2+}\) increased to 10 to 15 minutes. Ca\(^{2+}\) (1 mmol/L) added to unstimulated platelets resulted in only a minor increase in fura-2 ratio (Figure 2A), indicating that leakage of the dye out of platelets and passive thrombin-independent Ca\(^{2+}\) influx were both negligible. Thrombin also elicited Mn\(^{2+}\) influx, measured as the rate of quenching of fura-2 fluorescence (Figure 1C and 1D). Similar to Ca\(^{2+}\) influx, the rate of Mn\(^{2+}\) influx depended on the time after thrombin application. The Mn\(^{2+}\) influx was maximal after 30 s; however, after 5 minutes, thrombin caused almost no increase in the rate of Mn\(^{2+}\) influx as compared with the passive leak (Figure 2C).

BHQ-Induced Cation Influx
In Ca\(^{2+}\)-free medium, BHQ caused a slower, smaller, and more sustained increase in Ca\(^{2+}\) cyt (Figure 3B) compared with that of thrombin. Subsequent addition of external Ca\(^{2+}\) caused a rapid increase in Ca\(^{2+}\) cyt which, after reaching a peak, declined to a steady-state level (Figure 3A and 3B). Unlike the thrombin-activated Ca\(^{2+}\) influx, the amplitude of the BHQ-induced Ca\(^{2+}\) influx increased with time following application of BHQ (Figures 2B and 3), reaching a maximum after 5 to 10 minutes.

As with Ca\(^{2+}\) influx, BHQ-induced Mn\(^{2+}\) influx increased during the period between the addition of BHQ and Mn\(^{2+}\), being negligible during the first 30 s and reaching a maximum in 5 minutes. Figure 2D summarizes the relationship between the length of the period between addition of BHQ and Mn\(^{2+}\) and the rate of influx.

Effect of NO on Thrombin- and BHQ-Activated Ca\(^{2+}\) and Mn\(^{2+}\) Influx
To determine whether authentic NO has an inhibitory effect on capacitative cation influx in human platelets, NO was applied 30 s before addition of Ca\(^{2+}\) or Mn\(^{2+}\) in platelets stimulated with thrombin or BHQ. When added after thrombin-induced Ca\(^{2+}\) mobilization had reached the peak, NO (1 mmol/L) significantly increased the initial rate of decline in Ca\(^{2+}\) cyt (from 0.007±0.002 ratio units/s before NO addition to 0.088±0.004 ratio units/s after NO addition; n=6, P<0.001) (Figure 1A; see also Figure 4C) and profoundly inhibited the subsequent Ca\(^{2+}\) rise and Mn\(^{2+}\) influx (Figure 1). The effect of NO did not depend on the...
time of incubation with thrombin (Figure 2A and 2C), decreasing Ca\(^{2+}\) concentration and Mn\(^{2+}\) influx to a level that approximated the level that could be attributed to passive Ca\(^{2+}\) leak. When added to the thrombin-stimulated cells after Ca\(^{2+}\) influx reached equilibrium, NO decreased Ca\(^{2+}\)\(_{\text{cyt}}\) to a level similar to that reached when NO was added before Ca\(^{2+}\) (Figure 1A and 1B). When NO-containing solution was exposed to the air for 1 hour and then applied to platelets, it produced no effect on Ca\(^{2+}\)\(_{\text{cyt}}\) (not shown, n=3), indicating that NO, rather than the products of its degradation, was the active inhibitor of thrombin-induced cation influx.

Contrary to that induced by thrombin, the influx of Ca\(^{2+}\) and Mn\(^{2+}\) induced by BHQ was affected by NO only during the first few minutes of BHQ treatment (Figure 3A and 3C). Summary data are shown in Figure 2B and 2D. However, after pretreatment of platelets with BHQ for 5 to 10 minutes, NO did not affect the subsequent Ca\(^{2+}\) and Mn\(^{2+}\) influx (Figures 2B and 2D and 3B and 3D) significantly. These results illustrate the time-dependent disappearance of the effect of NO on capacitative cation influx during the progressive inhibition of SERCA by BHQ. This points to the importance of SERCA-dependent store refilling for NO-induced inhibition of influx.

**Figure 4.** Effect of NO on the refilling of ionomycin-releasable Ca\(^{2+}\) stores. In the absence of extracellular Ca\(^{2+}\), ionomycin (2 \(\mu\)mol/L) was applied alone (A, Iono trace) or 5 minutes after thrombin (0.5 U/mL) (A through D, control trace) to release all stored Ca\(^{2+}\). NO (1 \(\mu\)mol/L) was added 30 s before (B, + NO trace), 1 minute after (C, + NO trace), or 4.5 minutes after thrombin (D, + NO trace).

**NO Increases the Amount of Ca\(^{2+}\) Released by Ionomycin in Thrombin-Activated Platelets**

We conducted the following experiments to determine whether NO affects Ca\(^{2+}\) stores in thrombin-activated platelets. Figure 4 shows thrombin-induced Ca\(^{2+}\) release in the absence of extracellular Ca\(^{2+}\). Five minutes after thrombin application, ionomycin (2 \(\mu\)mol/L) was applied to release the remaining portion of Ca\(^{2+}\) from the stores. When ionomycin was applied after thrombin, it caused a 2.5 times smaller rise in Ca\(^{2+}\)\(_{\text{cyt}}\) than when it was applied alone to unstimulated platelets (Figure 4A; peak \(\Delta R_{340/380}\) was 1.4±0.1 versus 3.5±0.3 ratio units, respectively; n=4, P<0.005). This indicates that after release from the stores by thrombin, Ca\(^{2+}\) is taken up into the stores and is also extruded from the cells. To test whether NO can increase the amount of Ca\(^{2+}\) in the stores after they were emptied by thrombin, NO (1 \(\mu\)mol/L) was added at different times before or after the agonist. When NO was applied 30 s before thrombin (Figure 4B), the amplitude of the thrombin response was slightly suppressed (peak \(\Delta R_{340/380}\) was 1.0±0.1 versus 1.3±0.1 ratio units in control; n=4, P>0.05), but the initial rate of decline in Ca\(^{2+}\)\(_{\text{cyt}}\) was significantly accelerated (from 0.007±0.001 to 0.014±0.001 ratio units/s; n=4, P<0.005). The amplitude of the ionomycin-induced Ca\(^{2+}\) release in this case was ≈65% more than that obtained without NO (peak \(\Delta R_{340/380}\) was 2.2±0.1 compared with 1.4±0.1 ratio units without NO; n=4, P<0.001). When added 1 minute after thrombin, NO greatly accelerated the decline in Ca\(^{2+}\)\(_{\text{cyt}}\) and increased the response to ionomycin by 36% (peak \(\Delta R_{340/380}\) was 1.7±0.1 ratio units; n=4, P<0.05; Figure 4C). When NO was applied 30 s before ionomycin, it did not significantly increase the amplitude of Ca\(^{2+}\) release by ionomycin (peak \(\Delta R_{340/380}\) was 1.5±0.1 ratio units; n=4, P>0.05; Figure 4D).

These results suggest that after Ca\(^{2+}\) is released from the stores by thrombin, NO can promote its uptake into the stores, which can be a result of acceleration of Ca\(^{2+}\) back-sequestration or, alternatively, inhibition of Ca\(^{2+}\) release. To discriminate between these possibilities, the following experiments were performed.

**Inhibition of SERCA Abolishes the Effect of NO on Thrombin-Induced Ca\(^{2+}\) Release**

In Ca\(^{2+}\)-free medium, thrombin (0.5 U/mL) caused a fast Ca\(^{2+}\) rise resulting from Ca\(^{2+}\) release from the stores, followed by a slower Ca\(^{2+}\) decline as a result of its uptake into the stores and extrusion from the cell (Figure 5A). Addition of NO (1 \(\mu\)mol/L) 30 s before thrombin (Figure 5A) decreased the peak of transient Ca\(^{2+}\) rise (from 1.4±0.1 ratio units to 0.9±0.1; n=5, P<0.05) and dramatically accelerated the uptake/extrusion phase of Ca\(^{2+}\) transient (0.014±0.002 ratio units/s in the presence of NO versus 0.008±0.001 in control; n=5, P<0.05). When platelets were pretreated with 30 \(\mu\)mol/L BHQ for 5 minutes, Ca\(^{2+}\) was partially released from the stores, and thrombin was applied after BHQ released the remaining Ca\(^{2+}\) (Figure 5B). Pretreatment with BHQ eliminated the effect of NO on both peak thrombin–induced Ca\(^{2+}\) release (0.9±0.1 ratio units in control versus 0.9±0.1 in the presence of NO; n=5, P>0.05) and the rate of the subsequent uptake/extrusion (0.008±0.001 ratio units/s ver-
Figure 5. Effect of BHQ on the inhibition of thrombin-induced Ca\(^{2+}\) mobilization by NO. Thrombin (0.5 U/mL) was applied in Ca\(^{2+}\)-free medium in the absence (control traces) or presence (+NO traces) of NO (1 \(\mu\)mol/L applied at the time indicated) to the control platelets (A) or to the platelets in the presence of 30 \(\mu\)mol/L BHQ (B).

Inhibition of Mitochondria Does Not Prevent the Effect of NO on Ca\(^{2+}\) Influx

By taking up Ca\(^{2+}\) during agonist-induced activation, mitochondria could be involved in the regulation of capacitative Ca\(^{2+}\) influx in different types of cells. Inhibitors of mitochondrial metabolism were therefore used to determine whether mitochondria are involved in the inhibition of the thrombin-induced Ca\(^{2+}\) rise in intact platelets caused by NO. Figures 6A and 6B illustrate the effect of NO on thrombin-activated Ca\(^{2+}\) release and influx under control conditions (Figure 6A) and in the presence of the mitochondrial inhibitors (Figure 6B) oligomycin (1 \(\mu\)g/mL) and antimycin (2 \(\mu\)g/mL). As in the data obtained in the Jurkat cell line, inhibition of mitochondria decreased the amplitude of thrombin-induced Ca\(^{2+}\) rise (from 1.8±0.2 to 1.4±0.1; \(n=3\), \(P<0.05\)). However, the inhibition by NO of the thrombin-initiated Ca\(^{2+}\) release and influx was unchanged. These results indicate that uptake of Ca\(^{2+}\) into mitochondria is not involved in the inhibitory effect of NO on Ca\(^{2+}\)\(_{\text{cyt}}\) in platelets.

Inhibitor of cGMP Production, H-(1,2,4) Oxadiazolo(4,3-a) Quinoxallin-1-one (ODQ), Attenuates but Does Not Prevent the Effect of NO on Ca\(^{2+}\) Influx

To evaluate the role of cGMP formation in the effect of NO on Ca\(^{2+}\) influx, specific guanylate cyclase inhibitor, ODQ, was used. At the concentration used in these experiments, ODQ has been shown to completely prevent the NO-induced cGMP rise in platelets. The effect of NO (1 \(\mu\)mol/L) on the rate of Ca\(^{2+}\) removal from the cytoplasm and on Ca\(^{2+}\) influx was compared in control platelets (preincubated with the vehicle, 0.1% DMSO) and in the platelets preincubated with ODQ (10 \(\mu\)mol/L) for 1 hour (Figure 6C and 6D). Pretreatment with ODQ did not alter the kinetics of the Ca\(^{2+}\)-release phase or amplitude of the maximal Ca\(^{2+}\) influx following platelet stimulation with thrombin. However, it resulted in a small but significant attenuation of the inhibitory effect of NO on the Ca\(^{2+}\) increase associated with Ca\(^{2+}\) influx. The rates of the NO-induced acceleration of the falling phase of Ca\(^{2+}\) transient in control and ODQ-pretreated platelets were 0.051±0.003 ratio units/s and 0.039±0.003, respectively (\(n=5\), \(P<0.05\)), which corresponds to a 24% decrease. The amplitude of the thrombin-induced Ca\(^{2+}\) influx phase was inhibited by NO by 82% in the cells preincubated with vehicle (1.46±0.17 ratio units versus 0.26±0.02; \(n=5\), \(P<0.01\)) and only by 67% in ODQ-treated cells (1.44±0.16 ratio units versus 0.47±0.06; \(n=5\), \(P<0.01\)). Thus, the effect of NO (1 \(\mu\)mol/L) on Ca\(^{2+}\) influx in platelets is partially mediated by cGMP formation. These experiments also indicate the possible existence of another, cGMP-independent mechanism of NO-induced inhibition of capacitative cation influx in platelets.

Figure 6. Effects of inhibitors of mitochondria (A and B) or soluble guanylate cyclase (C and D) on the NO-induced inhibition of Ca\(^{2+}\) influx and acceleration of Ca\(^{2+}\) removal from the cytoplasm. Thrombin (0.5 U/mL) was applied in the absence of extracellular Ca\(^{2+}\), and then Ca\(^{2+}\) (1 \(\mu\)mol/L) was added to evoke Ca\(^{2+}\) influx in control platelets (A) or in platelets pretreated with oligomycin (1 \(\mu\)g/mL) and antimycin (2 \(\mu\)g/mL) for 2 minutes (B, control traces). Platelets were preincubated for 1 hour with 0.1% DMSO (C) or with 10 \(\mu\)mol/L ODQ (D). NO (1 \(\mu\)mol/L) was applied as shown after thrombin (+NO traces).

NO Accelerates Ca\(^{2+}\) Uptake by the Stores in Permeabilized Platelets

To directly monitor the process of filling and emptying of internal Ca\(^{2+}\) stores and to eliminate the involvement of the IP\(_{3}\) cascade and plasma membrane-associated regulatory proteins (plasma membrane Ca\(^{2+}\)-ATPase [PMCA] or Na\(^+/Ca\(^{2+}\) exchanger), the free-Ca\(^{2+}\) level was measured in suspensions of permeabilized platelets. Addition of saponin (20 \(\mu\)g/mL) to the platelets resuspended in cytoplasmic solution caused an immediate decrease in free Ca\(^{2+}\) (Figure 7A), which was completely prevented by BHQ (30 \(\mu\)mol/L) added 2 minutes before saponin (not shown). In the presence of 2 mmol/L ATP, cytoplasmic Ca\(^{2+}\) decreased from 1 to 3 \(\mu\)mol/L to a stable level or set point of 200 to 250 nmol/L and remained at the same low level for at least 10 minutes. When Ca\(^{2+}\) (1 \(\mu\)mol/L) was added to permeabilized cells, it was rapidly taken up by the stores, and the same level of free Ca\(^{2+}\) was restored (Figure 7A). This decrease in free Ca\(^{2+}\) concentration was used in our experiments as a measure of Ca\(^{2+}\)\(_{\text{cyt}}\).
uptake by the stores. Application of IP₃ (250 μmol/L) caused the rapid release of stored Ca²⁺, which also was almost completely resequestered into the stores. Inhibition of SERCA with BHQ caused slow and irreversible release of Ca²⁺ from the stores into the cytoplasmic solution. Heparin, a blocker of IP₃ receptors, completely abolished IP₃-induced Ca²⁺ release but was without any effect on the Ca²⁺ rise and uptake after exogenously added Ca²⁺ (Figure 7B), indicating that these processes do not involve IP₃-sensitive channels.

NO (1 μmol/L) significantly increased the rate of Ca²⁺ uptake after IP₃-induced release (0.077±0.006 versus 0.024±0.003 ratio units/s in control; n=5, P<0.001; Figure 7C), but it had no effect if applied after BHQ (Figure 7D). Because the effect of NO on IP₃-induced Ca²⁺ release could be due either to inhibition of IP₃-induced Ca²⁺ release or to acceleration of uptake into the stores, the effect of NO on Ca²⁺ sequestration in the absence of IP₃ was determined.

Exogenous Ca²⁺ was added to the suspension of platelets after the cells were permeabilized and the set point was allowed to reestablish. The rate of uptake of exogenously added Ca²⁺, as well as the lowest free-Ca²⁺ level reached after Ca²⁺ addition and uptake, was highly dependent on the ATP concentration (Figure 8A and 8B). When added 30 s before Ca²⁺, NO significantly accelerated the rate of Ca²⁺ sequestration into the stores (Figure 8C). However, the effect of NO was strongly dependent on the rate of sequestration observed in the absence of NO, which, in turn, depended on ATP concentration (Figure 8C and 8D). For example, at the higher rate of Ca²⁺ uptake typical for 2 mmol/L ATP (Figure 8B), there was no effect of NO (Figure 8D; 0.042±0.005 ratio units/s under control conditions and 0.044±0.006 ratio units/s in the presence of NO; n=4, P>0.1). However, at lower ATP concentrations (100 to 200 μmol/L ATP), NO significantly accelerated Ca²⁺ uptake (Figure 8C), but this was observed only in those experiments in which the control rate was lower than 0.03 ratio units/s (Figure 8E). For these experiments, NO significantly increased the rate of Ca²⁺ uptake into the stores from 0.015±0.003 in control to 0.022±0.003 ratio units/s in the presence of NO (n=7, P<0.05), representing an average 47% increase. Figure 8E summarizes the dependence of the effect of NO on the control rate of Ca²⁺ uptake measured in platelets from different platelet donors at 3 different ATP concentrations (100 μmol/L, 200 μmol/L, and 2 mmol/L). At low concen-
trations of ATP, when only part of the added Ca\textsuperscript{2+} was taken into the stores (Figure 8A and 8B), NO also increased the percentage of Ca\textsuperscript{2+} uptake from 49±11 to 68±8 (n=7, P<0.01). Thus, NO was able to increase the rate and quantity of Ca\textsuperscript{2+} uptake into the stores, but only when the uptake was submaximal under our experimental conditions.

**Discussion**

Recently, we showed that NO indirectly inhibits store-operated cation influx in vascular smooth muscle cells and proposed a hypothesis that this inhibition is a result of NO-induced acceleration of Ca\textsuperscript{2+} back-sequestration into the stores.\textsuperscript{32} Here we tested this idea in human platelets and provided new evidence showing that NO inhibits capacitative cation influx by accelerating the refilling of the stores, predominantly through increased SERCA-dependent Ca\textsuperscript{2+} uptake.

**Nonselective Cation Influx in Platelets Depends on the Stores**

Our data are consistent with a close correlation of cation influx in human platelets with the filling state of the stores.\textsuperscript{4–6} Thrombin causes a fast release of Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores in platelets, which is followed by partial back-sequestration into the stores and Ca\textsuperscript{2+} extrusion from cells via PMCA and Na\textsuperscript{+)/Ca\textsuperscript{2+}} exchanger.\textsuperscript{33} Under our experimental conditions Ca\textsuperscript{2+} and Mn\textsuperscript{2+} influx is maximally activated during the first 30 s after thrombin application and then gradually decreases. This decrease most probably results from the time-dependent partial back-sequestration of Ca\textsuperscript{2+} into the stores. However, we cannot rule out the involvement of other possible reasons, including desensitization of the thrombin receptor signaling cascade or inactivation of plasma membrane cation channels.

When Ca\textsuperscript{2+} was released slowly with BHQ (as was seen to occur in permeabilized platelets in Figure 7D), the amplitude of Ca\textsuperscript{2+} influx as well as the rate of Mn\textsuperscript{2+} influx increased gradually with time, reaching a maximum after 5 minutes (Figure 2B and 2D). The absence of back-sequestration of Ca\textsuperscript{2+} in the presence of BHQ might explain why the maximal amplitude of the BHQ-induced Ca\textsuperscript{2+} rise was 2.5 times higher than that induced by thrombin (Figure 2A and 2B). The rates of Mn\textsuperscript{2+} influx in thrombin- and BHQ-treated platelets were comparable (Figure 2C and 2D), suggesting that SERCA-dependent Ca\textsuperscript{2+} back-sequestration into the stores in the presence of thrombin, which is absent in BHQ-treated cells, can effectively reduce Ca\textsuperscript{2+} influx. Thus, the maximal Ca\textsuperscript{2+} and Mn\textsuperscript{2+} influx in platelets coincides with the maximal emptying of the stores, consistent with a mechanism of capacitative cation influx.

**NO Inhibits Capacitative Cation Influx in Human Platelets, but SERCA-Dependent Store Refilling Is Required**

Our data indicate that SERCA-dependent refilling of the stores is required for NO to inhibit Ca\textsuperscript{2+} and Mn\textsuperscript{2+} influx. When Ca\textsuperscript{2+}/Mn\textsuperscript{2+} influx was activated by thrombin, NO was very effective and blocked this influx almost completely and independently of the time after thrombin application (Figures 1 and 2). In BHQ-treated platelets, however, NO was able to partially inhibit Ca\textsuperscript{2+} and Mn\textsuperscript{2+} influx and the rise in Ca\textsuperscript{2+}, only during the first few minutes after BHQ application. During this initial phase, presumably, SERCA is only partially inhibited and some back-sequestration of Ca\textsuperscript{2+} into the stores can still occur. After 5 to 10 minutes, when the process of SERCA inhibition by BHQ is apparently complete and maximal cation influx has developed (Figure 2), the inhibitory effect of NO on Ca\textsuperscript{2+}, disappears. The absence of the inhibitory effect of NO on maximum Ca\textsuperscript{2+} and Mn\textsuperscript{2+} influx after a 10-minute treatment with BHQ suggests that NO does not have a direct effect on plasma membrane cation channels, which are responsible for such influx.

The time-dependent loss of the effect of NO on cation influx demonstrated in the presence of the SERCA inhibitor could explain why some researchers\textsuperscript{18,34} failed to detect the inhibition of TG-induced cation influx in platelets by NO donors, while others\textsuperscript{17} reported such an inhibition. The variability in the effect of NO could be also related to the incomplete inhibition of all SERCA isoforms by TG under different experimental conditions or to the differences between BHQ- and TG-sensitive Ca\textsuperscript{2+} stores and their role in the regulation of cation channels.

**NO Promotes SERCA-Dependent Back-Sequestration of Ca\textsuperscript{2+} Into the Stores**

The absence of an effect of NO on capacitative cation influx when SERCA is inhibited shows that NO does not inhibit the influx directly and suggests the importance of functional SERCA for the NO-induced inhibition of cation influx. To determine whether NO indeed can accelerate the refilling of Ca\textsuperscript{2+} stores in a SERCA-dependent manner, the following 2 approaches were used. In the first approach, ionomycin was used at a high concentration to release Ca\textsuperscript{2+} from all the stores to determine whether NO can promote the refilling of the stores emptied by thrombin. In thrombin-activated platelets, NO was shown to significantly increase the total intracellular Ca\textsuperscript{2+} pool released by ionomycin. The effect of NO was maximal if it was added before initiation of Ca\textsuperscript{2+} release by thrombin, apparently because it promotes back-sequestration of Ca\textsuperscript{2+} released by IP\textsubscript{3}. When NO was added later, most of the released Ca\textsuperscript{2+} was already removed from the cytoplasm, which explains the insignificant increase in the Ca\textsuperscript{2+} stores (Figure 4).

Permeabilized platelets were used in the second approach, which allows direct observations of the Ca\textsuperscript{2+} movements in and out of intracellular stores. Using this method, NO was found to accelerate uptake of Ca\textsuperscript{2+} either after it was released from the stores with IP\textsubscript{3} or after exogenous Ca\textsuperscript{2+} was added (Figure 8). The effect of NO disappeared in the presence of BHQ. The strong dependence on ATP concentration of Ca\textsuperscript{2+} uptake in permeabilized platelets (Figure 8) points to the involvement of an ATP-using process, which, most logically, is SERCA. Importantly, the effect of NO strongly depends on the initial rate of Ca\textsuperscript{2+} uptake (Figure 8). At a maximal rate of Ca\textsuperscript{2+} sequestration, the possibility of a further increase of SERCA activity by NO is unlikely. Under physiological conditions, one would expect SERCA to work at a lower, submaximal rate that would allow its further acceleration by
NO. In permeabilized platelets, NO had a stimulatory effect only when the rate of Ca\(^{2+}\) uptake was experimentally decreased (by decreasing the concentration of ATP) (Figure 8C and 8E). These results provide a possible explanation of the fact that some investigators did not observe an effect of NO on SERCA.\(^{35}\) Indeed, SERCA activity is usually measured under experimental conditions providing its highest activity (at high Ca\(^{2+}\) and/or ATP concentrations), which might exclude its further acceleration by NO.

Role of IP\(_3\)-Induced Ca\(^{2+}\) Release, PMCA, Na\(^+\)/Ca\(^{2+}\) Exchanger, and Mitochondria in the Effect of NO on Ca\(^{2+}\)\(_{\text{eryt}}\) in Human Platelets

NO has been shown to inhibit the transient Ca\(^{2+}\) rise caused by agonists in a variety of cells, including platelets.\(^{14,16}\) which theoretically can be mediated through NO-induced inhibition of IP\(_3\) production by phospholipase C or suppression of IP\(_3\)-induced Ca\(^{2+}\) release, on one hand, and a rapid acceleration of Ca\(^{2+}\) back-sequestration into the stores, on the other hand.

Although we cannot completely exclude an effect of NO exerted by changes in the production or action of IP\(_3\), there are several reasons why back-sequestration of Ca\(^{2+}\) into the stores by SERCA appears to be the major physiological mechanism for the NO-induced decrease in Ca\(^{2+}\)\(_{\text{eryt}}\) in human platelets. First, inhibition of SERCA with BHQ (which prevents Ca\(^{2+}\) back-sequestration, but not IP\(_3\)-induced Ca\(^{2+}\) release) completely abolished the effect of NO on the thrombin-induced transient Ca\(^{2+}\) rise (Figure 5). Second, even without involvement of IP\(_3\) in permeabilized platelets, NO significantly increased uptake of externally added Ca\(^{2+}\), accelerating the rate of uptake and lowering the free Ca\(^{2+}\) level (Figure 8). Third, the effect of NO depended on ATP concentration (Figure 8), pointing to the regulation of an ATP-dependent process such as SERCA.

PMCA and Na\(^+\)/Ca\(^{2+}\) exchanger have been proposed to be targets for NO action in other cells (for review, see Reference 36). In human platelets, we found no evidence for a significant role of these Ca\(^{2+}\)-removal mechanisms in the effects of NO. Indeed, when SERCA was inhibited by BHQ, Ca\(^{2+}\) (released by thrombin) was successfully removed from the cytoplasm (Figure 5), pointing to the activity of PMCA, Na\(^+\)/Ca\(^{2+}\) exchanger, or both. However, NO did not affect Ca\(^{2+}\) removal when SERCA was inhibited. Also, there was no effect of NO on the peak Ca\(^{2+}\) rise in BHQ-treated platelets, although such extrusion mechanisms as PMCA and Na\(^+\)/Ca\(^{2+}\) exchanger are expected to work under these conditions.

By taking up Ca\(^{2+}\) during agonist-induced activation, mitochondria could be involved in the regulation of capacitative Ca\(^{2+}\) influx in different types of cells.\(^{21-23}\) Our experiments with mitochondrial inhibitors (Figure 6) show that the effect of NO on intracellular Ca\(^{2+}\) does not depend on oxidative metabolism by, or Ca\(^{2+}\) uptake into, mitochondria.

Thus, IP\(_3\)-induced Ca\(^{2+}\) release, PMCA, Na\(^+\)-Ca\(^{2+}\) exchanger, and mitochondria, although they are potential targets for NO, seem not to play an important role in the effects of NO on Ca\(^{2+}\)\(_{\text{eryt}}\) in human platelets.

Role of cGMP Production in the Effect of NO on Ca\(^{2+}\) Influx in Human Platelets

It is well known that NO\(^{27}\) and NO donors\(^{27,38}\) stimulate cGMP production in human platelets leading to the activation of cGMP-dependent protein kinase and inhibition of agonist-induced Ca\(^{2+}\)\(_{\text{eryt}}\) rise and platelet aggregation.\(^{37,39}\) In rat aortic smooth muscle cells, this effect was partially attributed to the phosphorylation of the SERCA-regulatory protein phospholamban and thus the increased sensitivity of SERCA to Ca\(^{2+}\).\(^{40}\) It is tempting to look for a similar explanation for the effect of NO in platelets. In fact, a phospholamban-like 22-kDa protein, thrombolamban, found in platelets,\(^{41}\) has been shown to regulate the activity of Ca\(^{2+}\) uptake by the ER vesicles. However, comparative analysis of phospholamban and thrombolamban revealed differences in their structural and physical properties, as well as in the mechanisms for activation of the respective Ca\(^{2+}\) pumps.\(^{42}\) Moreover, no phosphorylation of thrombolamban by sodium nitroprusside or cGMP analogs was found in platelets.\(^{39,43}\) Thus, it seems unlikely that cGMP-dependent stimulation of platelet SERCA by NO is mediated by thrombolamban.

Inhibition of guanylate cyclase by ODQ has been shown to eliminate the cGMP rise caused by NO donors and to prevent their effect on platelet aggregation.\(^{27}\) However, there are some indications that cGMP might not account for all the effects of authentic NO on Ca\(^{2+}\)\(_{\text{eryt}}\). It was shown recently that inhibition of cGMP production with ODQ suppresses, but does not eliminate, the effects of authentic NO on intracellular Ca\(^{2+}\) in smooth muscle cells,\(^{44}\) as well as the effect of the NO donor S-nitrosglutathione in platelets.\(^{45}\) Similarly, we found that pretreatment of human platelets with 10 μmol/L ODQ for 1 hour only partially blocked the inhibitory effect of NO on capacitative Ca\(^{2+}\) influx (Figure 6). This result points to the possibility that along with a cGMP-dependent pathway there is also another, cGMP-independent mechanism of NO effect on Ca\(^{2+}\) influx in human platelets. Indeed, direct NO-induced nitrosylation of critical thiols is known to be responsible for cGMP-independent effects of NO on some other proteins.\(^{46-48}\) Further studies of the effects of NO on SERCA need to be done to fully understand this physiologically important pathway for regulation of capacitative cation influx and intracellular Ca\(^{2+}\) in platelets and other nonexcitable cells.

Acknowledgments

This work was supported by National Institutes of Health grants Specialized Center of Research HL55993 and R01 HL54150. We would like to thank Dr. E. Simons for help in establishing the platelet isolation procedures and Dr. B. Corkey for useful suggestions and comments on the experiments with permeabilized platelets.

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Nitric Oxide Inhibits Capacitative Cation Influx in Human Platelets by Promoting Sarcoplasmic/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase–Dependent Refilling of Ca\(^{2+}\) Stores

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*Circ Res.* 1999;84:201-209
doi: 10.1161/01.RES.84.2.201

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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