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 μmol/L 2,5-di-(tert-butyl)-1,4-benzohydroquinone (BHQ). NO (1 μmol/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 μmol/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 μmol/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

An 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\(^{2+}\)_cyt 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

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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. Prefilled 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 2.7, MgCl₂·6H₂O 1, NaN₃PO₄ 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 μU/mL 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 × 10⁹/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 ~10⁶/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 × 10⁶ 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⁺]cyt in platelets. PRP was diluted with standard buffer to the final cell number of 10⁸/mL that had been bubbled with nitrogen gas to remove oxygen.

**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⁺ influx with no elevation of IP₃,²⁰ 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⁺, 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 \(\mu\)mol/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

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**Figure 1.** Effect of NO on thrombin-induced Ca\(^{2+}\) (A and B) and Mn\(^{2+}\) (C and D) influx. \(F_{340}/F_{380}\) ratio (A and B) and \(F_{360}\) fluorescence (C and D; expressed as a percentage of \(F_{360}\) at the moment of Mn\(^{2+}\) addition) show the changes in Ca\(^{2+}\)\(_{cyt}\) and Mn\(^{2+}\) influx, respectively. Platelets were stimulated with thrombin (0.5 U/mL) in Ca\(^{2+}\)-free medium followed by the addition of Ca\(^{2+}\) (1 mmol/L; A and B) or Mn\(^{2+}\) (100 \(\mu\)mol/L; C and D) 1 minute (A and C) or 10 minutes (B and D) after thrombin application (control traces). NO (1 \(\mu\)mol/L) was applied 30 s before Ca\(^{2+}\) or Mn\(^{2+}\) addition (+NO traces). In A and B, NO was also applied to the control platelets at the time shown after Ca\(^{2+}\) influx reached a plateau.

**Figure 2.** Summary data from experiments illustrated in Figures 1 and 3 showing the time dependence of thrombin- and BHQ-induced Ca\(^{2+}\) and Mn\(^{2+}\) influx and the effect of NO. Data represent the maximal rise in Ca\(^{2+}\) after its addition to the extracellular medium (A and B) and the initial rate of Mn\(^{2+}\) influx (C and D) at different times after application of thrombin (A and C) or BHQ (B and D) in control cells (open bars), in the presence of NO (1 \(\mu\)mol/L added 30 s before Ca\(^{2+}\) or Mn\(^{2+}\); solid bars), and in unstimulated cells (crosshatched bars). Each bar is an average±SEM of 3 to 7 experiments. *P<0.01; **P<0.001.

**Figure 3.** Effect of NO on BHQ-induced Ca\(^{2+}\) (A and B) and Mn\(^{2+}\) (C and D) influx. Experiments are similar to those in Figure 1, but BHQ (30 \(\mu\)mol/L) was added to platelets instead of thrombin.
time of incubation with thrombin (Figure 2A and 2C),
decreasing Ca\textsuperscript{2+} concentration and Mn\textsuperscript{2+} influx to a level
that approximated the level that could be attributed to
passive Ca\textsuperscript{2+} leak. When added to the thrombin-stimulated
cells after Ca\textsuperscript{2+} influx reached equilibrium, NO decreased
Ca\textsuperscript{2+}\textsubscript{cyt} to a level similar to that reached when NO was
added before Ca\textsuperscript{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\textsuperscript{2+}\textsubscript{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\textsuperscript{2+}
and Mn\textsuperscript{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,
before pretreatment of platelets with BHQ for 5 to 10 minutes,
NO did not affect the subsequent Ca\textsuperscript{2+} and Mn\textsuperscript{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 progres-
sive 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\textsuperscript{2+} stores.** In the absence of extracellular Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} Released by
Ionomycin in Thrombin-Activated Platelets

We conducted the following experiments to determine
whether NO affects Ca\textsuperscript{2+} stores in thrombin-activated plate-
lets. Figure 4 shows thrombin-induced Ca\textsuperscript{2+} release in the
absence of extracellular Ca\textsuperscript{2+}. Five minutes after thrombin
application, ionomycin (2 \mu mol/L) was applied to release the
remaining portion of Ca\textsuperscript{2+} from the stores. When ionomycin
was applied after thrombin, it caused a 2.5 times smaller rise
in Ca\textsuperscript{2+}\textsubscript{cyt} than when it was applied alone to unstimulated
platelets (Figure 4A; peak \Delta R\textsubscript{340/380} was 1.4±0.1 versus
3.5±0.3 ratio units, respectively; n=4, P<0.005). This indi-
cates that after release from the stores by thrombin, Ca\textsuperscript{2+} is
taken up into the stores and is also extruded from the cells. To
test whether NO can increase the amount of Ca\textsuperscript{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\textsubscript{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\textsuperscript{2+}\textsubscript{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\textsuperscript{2+} release in this case was ≈65% more
than that obtained without NO (peak \Delta R\textsubscript{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\textsuperscript{2+}\textsubscript{cyt} and increased the response
to ionomycin by 36% (peak \Delta R\textsubscript{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\textsuperscript{2+} release by ionomycin (peak \Delta R\textsubscript{340/380} was 1.5±0.1 ratio
units; n=4, P>0.05; Figure 4D).

These results suggest that after Ca\textsuperscript{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\textsuperscript{2+} back-
sequestration or, alternatively, inhibition of Ca\textsuperscript{2+} release. To
discriminate between these possibilities, the following exper-
iments were performed.

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

In Ca\textsuperscript{2+}-free medium, thrombin (0.5 U/mL) caused a fast Ca\textsuperscript{2+}
rise resulting from Ca\textsuperscript{2+} release from the stores, followed by
a slower Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} was partially released from
the stores, and thrombin was applied after BHQ released the
remaining Ca\textsuperscript{2+} (Figure 5B). Pretreatment with BHQ
eliminated the effect of NO on both peak thrombin–induced
Ca\textsuperscript{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-

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**Figure 4.** Effect of NO on the refilling of ionomycin-releasable
Ca\textsuperscript{2+} stores. In the absence of extracellular Ca\textsuperscript{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\textsuperscript{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).
Figure 6. Effects of inhibitors of mitochondria (A and B) or soluble guanylate cyclase (C and D) on the NO-induced inhibition of Ca\textsuperscript{2+} influx and acceleration of Ca\textsuperscript{2+} removal from the cytoplasm. Thrombin (0.5 U/mL) was applied in Ca\textsuperscript{2+}-free medium in the absence (control traces) or presence (+NO traces) of NO (1 μmol/L applied at the time indicated) to the control platelets (A) or to the platelets in the presence of 30 μmol/L BHQ (B).

Inhibition of Mitochondria Does Not Prevent the Effect of NO on Ca\textsuperscript{2+} cytoplasmic stores

By taking up Ca\textsuperscript{2+} during agonist-induced activation, mitochondria could be involved in the regulation of capacitative Ca\textsuperscript{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\textsuperscript{2+} rise in intact platelets caused by NO. Figures 6A and 6B illustrate the effect of NO on thrombin-activated Ca\textsuperscript{2+} release and influx under control conditions (Figure 6A) and in the presence of the mitochondrial inhibitors (Figure 6B) oligomycin (1 μg/mL) and antimycin (2 μg/mL). As in the data obtained in the Jurkat cell line, inhibition of mitochondria decreased the amplitude of thrombin-induced Ca\textsuperscript{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\textsuperscript{2+} release and influx was unchanged. These results indicate that uptake of Ca\textsuperscript{2+} into mitochondria is not involved in the inhibitory effect of NO on Ca\textsuperscript{2+} cytoplasmic stores 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\textsuperscript{2+} Influx

To evaluate the role of cGMP formation in the effect of NO on Ca\textsuperscript{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 μmol/L) on the rate of Ca\textsuperscript{2+} removal from the cytoplasm and on Ca\textsuperscript{2+} influx was compared in control platelets (preincubated with the vehicle, 0.1% DMSO) and in the platelets preincubated with ODQ (10 μmol/L) for 1 hour (Figure 6C and 6D). Pretreatment with ODQ did not alter the kinetics of the Ca\textsuperscript{2+}-release phase or amplitude of the maximal Ca\textsuperscript{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\textsuperscript{2+} increase associated with Ca\textsuperscript{2+} influx. The rates of the NO-induced acceleration of the falling phase of Ca\textsuperscript{2+} transient in control and ODQ-pretreated platelets were 0.05±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\textsuperscript{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 μmol/L) on Ca\textsuperscript{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.

NO Accelerates Ca\textsuperscript{2+} Uptake by the Stores in Permeabilized Platelets

To directly monitor the process of filling and emptying of internal Ca\textsuperscript{2+} stores and to eliminate the involvement of the IP\textsubscript{3} cascade and plasma membrane-associated regulatory proteins (plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA) or Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger), the free-Ca\textsuperscript{2+} level was measured in suspensions of permeabilized platelets. Addition of saponin (20 μg/mL) to the platelets resuspended in cytoplasmic solution caused an immediate decrease in free Ca\textsuperscript{2+} (Figure 7A), which was completely prevented by BHQ (30 μmol/L) added 2 minutes before saponin (not shown). In the presence of 2 mmol/L ATP, cytoplasmic Ca\textsuperscript{2+} decreased from 1 to 3 μmol/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\textsuperscript{2+} (1 μmol/L) was added to permeabilized cells, it was rapidly taken up by the stores, and the same level of free Ca\textsuperscript{2+} was restored (Figure 7A). This decrease in free Ca\textsuperscript{2+} concentration was used in our experiments as a measure of Ca\textsuperscript{2+}.
uptake by the stores. Application of IP$_3$ (250 μmol/L) caused the rapid release of stored Ca$^{2+}$, which was almost completely resequestered into the stores. Inhibition of SERCA with BHQ caused slow and irreversible release of Ca$^{2+}$ from the stores into the cytoplasmic solution. Heparin, a blocker of IP$_3$ receptors, completely abolished IP$_3$-induced Ca$^{2+}$ release but was without any effect on the Ca$^{2+}$ rise and uptake after exogenously added Ca$^{2+}$ (Figure 7B), indicating that these processes do not involve IP$_3$-sensitive channels.

NO (1 μmol/L) significantly increased the rate of Ca$^{2+}$ uptake after IP$_3$-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 IP$_3$-induced Ca$^{2+}$ release could be due either to inhibition of IP$_3$-induced Ca$^{2+}$ release or to acceleration of uptake into the stores, the effect of NO on Ca$^{2+}$ sequestration in the absence of IP$_3$ was determined.

Exogenous Ca$^{2+}$ 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 Ca$^{2+}$, as well as the lowest free-Ca$^{2+}$ level reached after Ca$^{2+}$ addition and uptake, was highly dependent on the ATP concentration (Figure 8A and 8B). When added 30 s before Ca$^{2+}$, NO significantly accelerated the rate of Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ uptake into the stores from 0.015±0.003 ratio units/s 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 Ca$^{2+}$ 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-

Figure 7. Ca$^{2+}$ release and uptake in permeabilized platelets. Platelets were suspended in cytosolic solution containing fura-2.

A, Changes in free Ca$^{2+}$ are shown following application of saponin (20 μg/mL), external Ca$^{2+}$ (1 μmol/L), IP$_3$ (250 μmol/L), and BHQ (10 μmol/L) in the presence of 2 mmol/L ATP. B, The same experiment as in panel A is shown in the presence of heparin (40 μg/mL). C and D, Changes in free Ca$^{2+}$ in permeabilized platelets following application of IP$_3$ (100 μmol/L) or BHQ (10 μmol/L). NO (1 μmol/L) was applied at the times shown.

Figure 8. NO-induced acceleration of Ca$^{2+}$ uptake in permeabilized platelets. A, Ca$^{2+}$ transient (normalized to the maximal Ca$^{2+}$ rise) following addition of Ca$^{2+}$ (1 μmol/L) in the presence of 500 and 50 μmol/L ATP. B, The dependence on ATP concentration of the rate of initial Ca$^{2+}$ uptake (left scale) and the percentage of maximal Ca$^{2+}$ uptake (right scale). The rate of initial Ca$^{2+}$ uptake was estimated by linear fit during the first 20 s of the decline in free Ca$^{2+}$ in experiments such as the one shown in panel A. C and D, The decrease in Ca$^{2+}$ (normalized to the maximum rise) following application of external Ca$^{2+}$ (1 μmol/L) in the presence of 200 μmol/L ATP (C) or 2 mmol/L ATP (D) in the absence of NO (control) or presence of NO (+NO, 1 μmol/L added 30 s before Ca$^{2+}$). Dotted lines show the linear fit of the initial Ca$^{2+}$ uptake in the presence or absence of NO. E, The relationship between the initial rate of Ca$^{2+}$ uptake under control conditions at 100 μmol/L (C), 200 μmol/L (●), or 2 mmol/L (●) ATP and the uptake after addition of 1 μmol/L NO. One hundred percent is the rate of Ca$^{2+}$ uptake in the absence of NO. Each point represents a single experiment such as the one shown in panel C or panel D. The arrow indicates experiment shown in panel C.
trations of ATP, when only part of the added $\text{Ca}^{2+}$ was taken into the stores (Figure 8A and 8B), NO also increased the percentage of $\text{Ca}^{2+}$ uptake from 49±11 to 68±8 ($n=7$, $P<0.01$). Thus, NO was able to increase the rate and quantity of $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ back-sequestration into the stores.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 $\text{Ca}^{2+}$ 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.4–6 Thrombin causes a fast release of $\text{Ca}^{2+}$ from IP$_3$-sensitive $\text{Ca}^{2+}$ stores in platelets, which is followed by partial back-sequestration into the stores and $\text{Ca}^{2+}$ extrusion from cells via PMCA and Na$^+$/Ca$^{2+}$ exchanger.33 Under our experimental conditions $\text{Ca}^{2+}$ and Mn$^{2+}$ 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 $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ was released slowly with BHQ (as was seen to occur in permeabilized platelets in Figure 7D), the amplitude of $\text{Ca}^{2+}$ influx as well as the rate of Mn$^{2+}$ influx increased gradually with time, reaching a maximum after 5 minutes (Figure 2B and 2D). The absence of back-sequestration of $\text{Ca}^{2+}$ in the presence of BHQ might explain why the maximal amplitude of the BHQ-induced $\text{Ca}^{2+}$ rise was ≈2.5 times higher than that induced by thrombin (Figure 2A and 2B). The rates of Mn$^{2+}$ influx in thrombin- and BHQ-treated platelets were comparable (Figure 2C and 2D), suggesting that SERCA-dependent $\text{Ca}^{2+}$ back-sequestration into the stores in the presence of thrombin, which is absent in BHQ-treated cells, can effectively reduce $\text{Ca}^{2+}$ influx. Thus, the maximal $\text{Ca}^{2+}$ and Mn$^{2+}$ 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 $\text{Ca}^{2+}$ and Mn$^{2+}$ influx. When $\text{Ca}^{2+}$/Mn$^{2+}$ 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 $\text{Ca}^{2+}$ and Mn$^{2+}$ influx and the rise in $\text{Ca}^{2+}$, only during the first few minutes after BHQ application. During this initial phase, presumably, SERCA is only partially inhibited and some back-sequestration of $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$, disappears. The absence of the inhibitory effect of NO on maximum $\text{Ca}^{2+}$ and Mn$^{2+}$ 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 researchers18–24 failed to detect the inhibition of TG-induced cation influx in platelets by NO donors, while others17 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 $\text{Ca}^{2+}$ stores and their role in the regulation of cation channels.

**NO Promotes SERCA-Dependent Back-Sequestration of $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ pool released by ionomycin. The effect of NO was maximal if it was added before initiation of $\text{Ca}^{2+}$ release by thrombin, apparently because it promotes back-sequestration of $\text{Ca}^{2+}$ released by IP$_3$. When NO was added later, most of the released $\text{Ca}^{2+}$ was already removed from the cytoplasm, which explains the insignificant increase in the $\text{Ca}^{2+}$ stores (Figure 4).

Permeabilized platelets were used in the second approach, which allows direct observations of the $\text{Ca}^{2+}$ movements in and out of intracellular stores. Using this method, NO was found to accelerate uptake of $\text{Ca}^{2+}$ either after it was released from the stores with IP$_3$ or after exogenous $\text{Ca}^{2+}$ was added (Figure 8). The effect of NO disappeared in the presence of BHQ. The strong dependence on ATP concentration of $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ uptake (Figure 8). At a maximal rate of $\text{Ca}^{2+}$ 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+}\(_{\text{cyt}}\) in Human Platelets**

NO has been shown to inhibit the transient Ca\(^{2+}\)\(_{\text{cyt}}\) 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+}\(_{\text{cyt}}\) 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\(^{2+}\)\(_{\text{cyt}}\) 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\(^{2+}\)\(_{\text{cyt}}\) (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\(^{2+}\)\(_{\text{cyt}}\) 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+}\(_{\text{cyt}}\) 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\(^{22,38}\) stimulate cGMP production in human platelets leading to the activation of cGMP-dependent protein kinase and inhibition of agonist-induced Ca\(^{2+}\(_{\text{cyt}}\) rise and platelet aggregation.\(^{7,35,39}\) 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+}\(_{\text{cyt}}\). 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-nitrosogluthathione in platelets.\(^{45}\) Similarly, we found that pretreatment of human platelets with 10 \(\mu\)mol/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.

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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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