Influence of authentic nitric oxide on basal cytosolic $[Ca^{2+}]$ and $Ca^{2+}$ release from internal stores in human platelets

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1 Nitric oxide (NO) donors inhibit platelet function and $Ca^{2+}$ mobilization evoked by different agonists. This led us to investigate the direct effects of authentic NO on basal cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]$) and on $Ca^{2+}$ mobilization induced by thrombin or by two inhibitors of intracellular $Ca^{2+}$-ATPases, thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone (t-BuBHQ).

2 Cytosolic $Ca^{2+}$ concentration was evaluated with Fura-2, in the absence of $Ca^{2+}$ influx. Addition of 5 $\mu$M NO increased by 48% the basal cytosolic $[Ca^{2+}]$ of resting human platelets whereas a lower concentration (0.1 $\mu$M) did not induce significant modifications. This NO-induced $Ca^{2+}$ increase was inversely correlated with the basal level of cytosolic $[Ca^{2+}]$.

3 NO pretreatment for 30 to 120 s decreased by 42 to 57% the transient $[Ca^{2+}]$, peak evoked by 0.10 uM thrombin and strongly attenuated the initial rate of $[Ca^{2+}]$, rise induced by 1 $\mu$M thapsigargin or by 20 $\mu$M t-BuBHQ. The two components of the thapsigargin response, the $Ca^{2+}$ release due to inhibition of $Ca^{2+}$ pumps and the thromboxane A$_2$-dependent self-amplification mechanism, were inhibited by NO. The observation that a subsequent stimulation was still capable of eliciting $Ca^{2+}$ release suggests the presence of NO-insensitive $Ca^{2+}$ stores.

4 These findings indicate that nitric oxide can modulate basal cytosolic $[Ca^{2+}]$ in unstimulated human platelets and decrease the $Ca^{2+}$ mobilization from NO-sensitive internal stores evoked by stimulation of recent agonists or by Ca$^{2+}$-ATPase inhibitors. This underlines the important role of nitric oxide in the modulation of platelet $Ca^{2+}$ handling.

Keywords: Human platelets; $[Ca^{2+}]$; nitric oxide; thrombin; thapsigargin; 2,5-di-(t-butyl)-1,4-benzohydroquinone (t-BuBHQ)

Introduction

Nitric oxide (NO) plays an important role in the regulation of vascular tone and blood pressure, in the neuronal transmission as well as in the apoptosis processes and in the modulation of immunological and inflammatory reactions (Moncada et al., 1991; Nathan, 1992; Schulz & Triggle, 1994; Lloyd-Jones & Bloch, 1996). Recently, it was shown that the nitric oxide pathway is present in human platelets (Radomski et al., 1990; Malinski et al., 1993; Muruganandam & Mutus, 1994; Zhou et al., 1995). NO was found to inhibit platelet aggregation through the activation of the soluble guanylate cyclase (Melion et al., 1981; Radomski et al., 1990). Until now, NO effects on platelet aggregation and/or $Ca^{2+}$ mobilization stimulated by different agonists have been studied by using NO-donors such as sodium nitroprusside (SNP), 3-morpholino-sydnoni- mine-hydrochloride (SIN-1) or nitroglycerin (NTG) (Melion et al., 1981; Gerzer et al., 1988; Astarie et al., 1992; Amano et al., 1994). These compounds released NO spontaneously or after transformation. The antiaggregatory properties and vasodilator action of these NO-donors were widely accepted for their beneficial effects, but the side effects produced by their intermediary or metabolized compounds are unknown. Up to now, there has been little information about the influence of authentic NO on $Ca^{2+}$ handling in human platelets.

To explore the mechanism by which NO affects $Ca^{2+}$ homeostasis in human platelets, we have investigated the direct effects of authentic NO on basal cytosolic $[Ca^{2+}]$ levels and its influence on $Ca^{2+}$ mobilization from internal stores, in the absence of $Ca^{2+}$ influx. To this end, we have compared the various types of $Ca^{2+}$ responses in human platelets induced by receptor occupancy versus inhibition of the Ca$^{2+}$-ATPase pump. Two structurally unrelated compounds, the sesquiterpene lactone tumour promoter, thapsigargin (Tg) and the benzohydroquinone derivative, 2,5-di-(t-butyl)-1,4-benzohydroquinone (t-BuBHQ), were used as specific inhibitors of the sarco/endoplasmic reticulum Ca$^{2+}$-ATPases (SERCA pumps) (Papp et al., 1992). These compounds elevate cytosolic $[Ca^{2+}]$ by inhibiting Ca$^{2+}$ reuptake into the dense tubular system (Thastrup, 1987; Thastrup et al., 1990).

Methods

Platelet preparation

Blood samples were collected between 08 h 00 min and 10 h 00 min from healthy subjects of either sex, aged between 20 and 45 years after an overnight fast. They had not taken any medications for at least 10 days. Venous blood was mixed with acid-citrate dextrose (ACD) anticoagulant at a ratio of 9 : 1 (vol/vol) (2.73% citric acid, 4.48% trisodium citrate and 2% glucose). Platelet-rich plasma (PRP) was obtained by centrifugation at a maximum acceleration of 530 g for 5 min at 20°C. All platelet measurements were performed within 3 h of blood sampling.

Nitric oxide measurement

NO standards were prepared by serial dilutions of saturated NO solutions, as previously described (Lantoine et al., 1995b). Saturated NO solutions were prepared under oxygen-free conditions by successive bubbling with argon for 20 min, then with pure NO gas (Aldrich) for 20 min. They were kept under NO atmosphere until use. Standards were made fresh for each experiment and kept in a glass flask with a thick rubber septum at 4°C. After the initial 20 min, the NO saturated solution was stable for more than 5 h and its concentration averaged 1–2 mM. Dilutions of the saturated solution were made with deoxygenated water. The concentrations of saturated and diluted NO solutions were assessed by spectrophotometric measurement with the Griess reagent (Green et al., 1982) and controlled by differential pulse amperometry with porphyrin-
and nafion-coated electrodes built in our laboratory (Lantoine et al., 1995a). All electrochemical studies were performed with the three-electrode potentiostatic system Biopulse made by Tacussel (Lantoine et al., 1995a).

**Measurement of platelet cytosolic Ca\(^{2+}\) concentration**

Platelet cytosolic [Ca\(^{2+}\)] was measured with the fluorescent indicator Fura-2, as described previously (Astarie-Dekuever et al., 1992). Platelets were loaded with 2 \(\mu\)M Fura-2AM for 40 min at 37°C in the presence of plasma. They were then washed by centrifugation at 270 \(g\) for 15 min at 20°C and resuspended at a density of \(1 \times 10^8\) cells ml\(^{-1}\) in a medium containing (in mM): NaCl 145, KCl 5, MgCl\(_2\) 0.5, glucose 5 and HEPES for rectified pH 7.4 at 37°C and 30 nM free Ca\(^{2+}\) concentration adjusted with the required Ca-EGTA buffer. Fluorescence intensities were measured at 37°C on a Spex Fluorolog CM111 (Instruments S.A., Paris, France) equipped with a 450W Xenon lamp, two excitation monochromators and a dual-mirror chopping mechanism that allows a rapid alternating excitation from 335 to 385 nm wavelengths, with a fixed emission wavelength of 510 nm. Each platelet sample was checked for Fura-2 loading by recording the fluorescence spectra. The fluorescence recording was systematically corrected for the autofluorescence of the cell suspension. Platelet cytosolic Ca\(^{2+}\) concentration was calculated from the experimental ratio of excitation fluorescence intensities \(R=I_1/I_2\) (Grynkiewicz et al., 1985). Nitric oxide, agonists, inhibitors and vehicles did not affect the fluorescence properties of the Fura 2-Ca and non Ca\(^{2+}\) forms.

**Materials**

Fura-2 acetoxyethyl ester (Fura-2AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.), SQ 295481 (5-Heptenoic acid, 7-[3-[2-[[(phenylamino) carbonyl] hydrazinyl] methyl]-7-oxabicyclo[2.2.1] hept-2-yl]) was from Cayman Chemical Company (Ann Arbor, MI, U.S.A.) and t-BuBHQ was from Calbiochem (Frankfurt, Germany). Thrombin and thapsigargin, and all other chemicals were from Sigma.

**Statistical analysis**

All data are expressed as means±s.e.mean. Experiments in which multiple groups were used were submitted to one-way analysis of variance (ANOVA) and differences between groups were assessed by paired Student’s \(t\) test. Correlation between two parameters was analysed by linear regression. \(P<0.05\) was considered to be statistically significant.

**Results**

**Effect of NO on the basal platelet cytosolic [Ca\(^{2+}\)]**

To determine the effect of NO on the Ca\(^{2+}\) intracellular stores, all the experiments were conducted in the presence of 30 nM external Ca\(^{2+}\) concentration that suppresses Ca\(^{2+}\) influx. Addition of 5 \(\mu\)M NO to unstimulated platelets increased the cytosolic [Ca\(^{2+}\)] by 48±7% (Figure 1a). This [Ca\(^{2+}\)] increase was rapid and stable for at least 120 s. It was observed to be negatively correlated with the basal level of platelet cytosolic [Ca\(^{2+}\)], indicating that the NO-induced Ca\(^{2+}\) increase was more marked when the initial resting level of platelet [Ca\(^{2+}\)] was low (Figure 1b). Addition of a lower NO concentration (0.1 \(\mu\)M) had no significant effect on the basal [Ca\(^{2+}\)] (Figure 1a).

**Effect of NO on the Ca\(^{2+}\) mobilization induced by thrombin**

In the absence of Ca\(^{2+}\) influx, 0.10 u ml\(^{-1}\) thrombin elevated the platelet cytosolic [Ca\(^{2+}\)] from the resting level (21±4 nM, \(n=10\)) to 216±19 nM, \(n=10\) within a few seconds. The [Ca\(^{2+}\)], peak then declined towards basal level (Figure 2a, trace 1). In the presence of 5 \(\mu\)M NO, the transient [Ca\(^{2+}\)] peak evoked by thrombin was reduced by 55±4% (Figure 2a (trace 2) and b). Cytosolic [Ca\(^{2+}\)] then returned to a similar level (41±4 vs 42±3 nM, \(n=10\), measured 150 s after the first stimulation, in the presence and absence of NO, respectively). Addition of 0.1 \(\mu\)M NO did not significantly modify the Ca\(^{2+}\) mobilization induced by thrombin (Figure 2b).

To investigate whether NO elicited a time-dependent discharge of Ca\(^{2+}\) stores, platelets were preincubated with 5 \(\mu\)M NO for 30, 60, 90 and 120 s before thrombin stimulation. In all cases, NO induced similar decreases in the transient [Ca\(^{2+}\)] peak, the amplitude of which (42 to 57%) did not vary with the incubation time (Figure 3).

When the thrombin signal had returned to their new equilibrium value, thapsigargin was able to release Ca\(^{2+}\) in the same order as the first stimulation, with a higher
transient [Ca\textsuperscript{2+}] peak in the presence of NO than it its absence (202±15 vs 175±15 nM for [Ca\textsuperscript{2+}], peak, n=4, \(P=0.09\)) and a significantly slower return towards low Ca\textsuperscript{2+} levels (85±3 vs 64±3 nM, measured 100 s after the second stimulation, n=4, \(P<0.001\)) (Figure 2a, traces 1 and 2).

**Effect of NO on the Ca\textsuperscript{2+} mobilization induced by thapsigargin**

Under the same experimental conditions, 1 \(\mu\)M thapsigargin, a concentration that maximally inhibits the Ca\textsuperscript{2+} re-uptake, raised Ca\textsuperscript{2+} from 25±3 nM to 255±12 nM (n=10). The time necessary to reach the [Ca\textsuperscript{2+}] peak was longer than 10 s. Cytosolic [Ca\textsuperscript{2+}] then stabilized at a level higher than the initial one (74±4 nM, n=10) (Figure 4a, trace 1). The transient [Ca\textsuperscript{2+}] peak induced by thapsigargin was reduced in platelets pretreated with 0.1 \(\mu\)M NO (187±5 vs 255±25 nM, n=4, \(P=0.045\)) and replaced by a slow progressive rise in those pretreated by 5 \(\mu\)M NO (Figure 4a, trace 2). Treatment with 0.1 or 5 \(\mu\)M NO decreased the thapsigargin-elicited initial rate of [Ca\textsuperscript{2+}] rise as indicated in Figure 4b.

Since thapsigargin affects platelet Ca\textsuperscript{2+} handling in part through the synthesis of thromboxane A\textsubscript{2} (TXA\textsubscript{2}) (Brune & Ullrich, 1991), platelets were treated with the specific thromboxane-receptor antagonist, SQ 29548. In platelets treated with 5 \(\mu\)M SQ 29548 which blocks the self-amplification mechanism, the transient [Ca\textsuperscript{2+}], response induced by thapsigargin was decreased by 50±2% (n=4, \(P<0.001\)) (Figure 4a, trace 3). Under these conditions, the thapsigargin-elicited Ca\textsuperscript{2+} release was comparable to that of 1 \(\mu\)M butylhydroxyquinoline, another inhibitor of sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (144±13 vs 135±3 nM, n=4 for each). Combined pretreatment with NO and SQ 29548 totally suppressed the transient [Ca\textsuperscript{2+}], peak evoked by thapsigargin, similar to that obtained with NO alone (Figure 4a, trace 4).

Stimulation by thrombin of thapsigargin-treated cells caused a dramatic decrease in Ca\textsuperscript{2+} release when compared to the first thapsigargin signal (Figure 4a, trace 1) or to that obtained with thrombin alone as the first signal (Figure 2a, trace 1). In contrast, in the presence of NO or SQ 29548 alone or the two combined, thrombin stimulation following thapsigargin treatment induced by a further significant Ca\textsuperscript{2+} release with a [Ca\textsuperscript{2+}] peak reaching 226±15 vs 88±6 nM, n=6, \(P<0.001\); 192±7 vs 92±5 nM, n=4, \(P<0.001\); 220±2 vs 96±5 nM, n=3, \(P<0.01\); for NO alone, SQ 29548 alone or both together, respectively (Figure 4a, traces 2, 3 and 4). The [Ca\textsuperscript{2+}] value measured 100 s after stimulation by thrombin always remained higher in platelets pretreated with NO than in the ones that were not, independently of a previous thromboxane A\textsubscript{2} receptor blockade by SQ 29548 (116±9 vs 76±7 nM, n=3, \(P=0.011\) and 115±6 vs 71±4 nM, n=6, \(P<0.001\), in the presence and absence of SQ 29548, respectively) (Figure 4a, traces 4 and 2).
To define the effect of NO on the modulation of the Ca^{2+} ATPase pump activities better, its influence on the effect of t-BuBHQ was investigated. As illustrated in Figure 5 (trace 1), 20 μM t-BuBHQ induced Ca^{2+}-release of the same amplitude as that evoked by the combined addition of thapsigargin and the thromboxane A_2 antagonist (Figure 4a, trace 3). Addition of 5 μM NO also suppressed the transient [Ca^{2+}]_i peak induced by t-BuBHQ (Figure 5, trace 2) and decreased the initial rate of [Ca^{2+}]_i rise (0.32±0.03 vs 24.02±2.08 nM s^{-1}, n=4, P=0.008).

Following the t-BuBHQ-induced Ca^{2+} transient, thrombin was able to release additional Ca^{2+} with the same amplitude as that obtained with thrombin alone, either in the presence or in the absence of NO (for [Ca^{2+}], peak: 179±23 vs 208±16 nM, n=3). Cytosolic [Ca^{2+}] then returned back to new equilibrium values that again remained elevated in platelets pretreated with NO (87±7 vs 77±6 nM, calculated at 100 s after thrombin stimulation, n=3, P=0.04) (Figure 5, traces 2 and 1).

**Discussion**

The present study was designed to investigate the effects of authentic NO on Ca^{2+} mobilization in human platelets. It demonstrates that, in the absence of Ca^{2+} influx, NO (i) increases basal cytosolic [Ca^{2+}] level in unstimulated platelets, (ii) attenuates the Ca^{2+} mobilization evoked either by thrombin and thromboxane A_2 or by thapsigargin and t-BuBHQ, the SERCA pump inhibitors, and (iii) blocks the self-amplification system involving thromboxane A_2 formation. To our knowledge, such effects of NO on Ca^{2+} homeostasis in intact human platelets have not previously been demonstrated.

The recent observation that NO inhibits the active Ca^{2+} uptake into platelet membrane vesicles (Pernollet et al., 1996) led us to check whether it could also increase the cytosolic Ca^{2+} concentration, as the well-known inhibitors of the SERCA pumps do (Thastrup, 1987; Thastrup et al., 1990; Papp et al., 1992). A significant rise in [Ca^{2+}], was indeed observed. It averaged 48% of the basal value and was more pronounced when the initial platelet [Ca^{2+}] level was low. A NO-mediated rise in the cytosolic [Ca^{2+}] has also been described in other cell types including intersitial cells from canine colon (Publicover et al., 1993), rat pancreatic β-cells (Willmott et al., 1995), intact sea urchin eggs (Willmott et al., 1996) and cultured nodose ganglion neurones from rabbits (Sato et al., 1996). In cells possessing
ryanodine-sensitive Ca\(^{2+}\) channels, this Ca\(^{2+}\) rise can be partially due to the production of cyclic ADP-ribose, a Ca\(^{2+}\) mobilizing agent (Publicover et al., 1993; Willmot et al., 1995; 1996). The presence of a ryanodine receptor mediated Ca\(^{2+}\)-induced mobilization of intracellular calcium from SR, which has not been established in platelets. The precise mechanism by which NO induced Ca\(^{2+}\) increase in human platelets remains therefore to be elucidated. The profile of the Ca\(^{2+}\) increase was markedly different from those observed after inhibition of SERCA pumps. It could be explained either by NO-induced concomitant reductions of the Ca\(^{2+}\) leak and Ca\(^{2+}\) pump activities, by a guanine 3'-5'-cyclic monophosphate (cyclicGMP)-dependent stimulation of the plasma membrane Ca\(^{2+}\) pump or by an, as yet unidentified NO-mobilized Ca\(^{2+}\) mobilizing pathway. A NO inhibitory effect on ATP production, similar to that found by Borutaiette & Brown (1996) can hardly be responsible for these alterations of Ca\(^{2+}\) movements and can be excluded, as indicated by the unchanged kinetics of Ca\(^{2+}\) return towards basal levels after thrombin stimulation (Figure 2a).

Under transient. Nositol mobilization induced by thapsigargin and t-BuBHQ. These two inhibitors of intracellular Ca\(^{2+}\)-ATPases elevated platelet cytosolic Ca\(^{2+}\) levels, but partially through different mechanisms. Thapsigargin produces a large Ca\(^{2+}\) signal due not only to Ca\(^{2+}\)-ATPase inhibition, but also to the subsequent synthesis of thromboxane A\(_2\) that triggers the phosphatidyli- nositol response (Brüne & Ulrich, 1991; Authi et al., 1993). As opposed, blockade of the thromboxane receptors by SQ 29548 attenuated by 50% the thapsigargin-induced Ca\(^{2+}\) transient. Under these conditions, the transient [Ca\(^{2+}\)]\(_i\) rise was similar to that induced by t-BuBHQ (Brüne & Ulrich, 1991; Astarie-Dekequer et al., 1995). We found that NO strongly influenced the Ca\(^{2+}\) responses induced by thapsigargin, irrespective of the presence of SQ 29548, and by t-BuBHQ. This indicates that NO alters the Ca\(^{2+}\) mobilization elicited by these two stimuli of Ca\(^{2+}\) responses to the direct inhibition of the Ca\(^{2+}\) pumps and to the subsequent formation of thromboxane A\(_2\). This agrees with the data showing that NO dose-dependently inhibits ATP-dependent Ca\(^{2+}\) uptake in platelet membrane vesicles (Pernollet et al., 1996) and suppresses thromboxane generation in thrombin-stimulated human platelets (Sane et al., 1989). The mechanism by which NO may directly or indirectly interfere with the SERCA pumps is unknown.

The presence of the thrombin-inhibited Ca\(^{2+}\)-induced mobilization of intracellular calcium from thapsigargin-treated cells was smaller than that observed when platelets were treated with both thapsigargin and SQ 29548, or with t-BuBHQ. This agrees with previous observations (Brüne & Ulrich, 1991; Astarie et al., 1995), indicating that thapsigargin mobilizes Ca\(^{2+}\) from thrombin-responsive intracellular stores when the self-amplification mechanism is intact. In platelets pretreated with NO, either in the presence of the absence of a thromboxane receptor antagonist the addition of thrombin as the second stimulus similarly released additional Ca\(^{2+}\) (Figure 4a, traces 2 and 4). This reinforces the above observation that nitric oxide mimics the effect of the thromboxane receptor antagonist or acts as an inhibitor of the cyclo-oxygenase-lipoxygenase pathway. In contrast, in platelets pretreated with thrombin, thapsigargin was still able to elicit a full Ca\(^{2+}\) increase, almost independent of NO pretreatment and of the extent of initial Ca\(^{2+}\) mobilization (Figure 2a, traces 1 and 2). This could be explained by two possibilities, either that NO exerts only a transient inhibition of Ca\(^{2+}\) pumps, or that the TXA\(_2\)-operated Ca\(^{2+}\) release from NO-insensitive stores becomes accessible in prestimulated platelets. However, NO pretreatment slowed down the return to basal levels of the addition of Ca\(^{2+}\) or thrombin as the second stimulus (Figures 2a, 4a and 5), suggesting that the inhibition of Ca\(^{2+}\) reuptake by SERCA pumps and/or that of Ca\(^{2+}\) efflux lead(s) to an enhanced cytosolic Ca\(^{2+}\) concentration.

The relatively high NO concentration used here as in the other studies (Publicover et al., 1993; Willmot et al., 1995; 1996; Sato et al., 1996) questions the physiological significance of these NO effects on the NO-elicted Ca\(^{2+}\) increase. When the actual NO concentration available in the platelet suspension was controlled throughout the experiment by a NO-sensitive electrode and differential pulse amperometry, it was found to decrease from 5 to 2 μM within seconds after addition, and then to disappear slowly with an apparent half-life of approximately 400 s (unpublished data). A low NO concentration (0.1 μM) was also capable of significantly decreasing the initial rate of thapsigargin-induced Ca\(^{2+}\) rise. This level resembles those produced by stimulated endothelial cells (Tsakarahar et al., 1993; Kanai et al., 1995) and could therefore be relevant.

In conclusion, nitric oxide increases basal [Ca\(^{2+}\)] in unstimulated platelets and decreases Ca\(^{2+}\) discharge from stores sensitive to receptor stimulation or to Ca\(^{2+}\)-ATPase inhibition. In addition, nitric oxide behaves as an inhibitor of the thromboxane A\(_2\) pathway responsible for the self-amplification system. A better characterization of the NO-sensitive Ca\(^{2+}\) pools should contribute to the understanding of the physiological regulation of platelet reactivity. All these NO properties could participate in its inhibitory effects on agonist-induced platelet release of vasoactive substances and growth factors, which participate in the development of intimal hyperplasia and atherosclerosis.

This study was in part supported by ACC SV9 and ACC SV11 grants from the Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la Recherche.

References


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