Kinetics of a Cellular Nitric Oxide/cGMP/Phosphodiesterase-5 Pathway*§

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Rat platelets served as a model to evaluate quantitatively how guanylate cyclase (GC)-coupled nitric oxide (NO) receptors and phosphodiesterases (here phosphodiesterase-5) interact to transduce NO signals in cells. The platelets expressed mRNA only for the α₁ and β₁ GC-coupled receptor subunits. In intact platelets, the potency of NO for elevating cGMP (EC₅₀ = 10 nM) was lower than in lysed platelets (EC₅₀ = 1.7 nM). The limiting activities of GC and phosphodiesterase in intact platelets were both very high, being equivalent to about 100 μM/s. With low phosphodiesterase activity (imposed by 100 μM sildenafil), the cGMP response over time was hyperbolic in shape for a range of NO concentrations or GC activities due to GC desensitization. Without a phosphodiesterase inhibitor, NO generated only brief cGMP transients, peaking after 2–5 s but amounting maximally to about 150 μM cGMP. The transients were caused partly by GC desensitization, which varied in rate (half-time up to 3 s) and extent (up to 80%) depending on the NO concentration, and partly by an enhancement of the phosphodiesterase catalytic activity with time, which was deduced to be up to 30-fold and to occur with a half-time of up to 5 s. The results were simulated by a quantitative model, which also explains the varied shapes of cGMP responses to NO found in other cells. Downstream phosphorylation in platelets was detectable within 2 s, and, with continuous exposure (1 min), this pathway could be engaged by subnanomolar NO concentrations (EC₅₀ = 0.5 nM).

Whereas the spatio-temporal dynamics of some of the primary steps of cellular signal transduction, such as synaptic transmission and changes in intracellular Ca²⁺, have become well understood in quantitative terms (1, 2), knowledge of downstream signaling cascades remains largely qualitative. Nevertheless, the kinetic properties of the second messenger cascades are likely to be similarly instrumental in determining how cells respond.

A case in point is nitric oxide (NO) signaling. NO acts as a diffusable chemical messenger throughout the body, where it subserves diverse functions, including smooth muscle relaxation, inhibition of platelet aggregation, and the induction of synaptic plasticity (3, 4). NO elicits these and other physiological effects by binding to its guanylyl cyclase (GC)-coupled receptors, thereby evoking the accumulation of cGMP in target cells. The receptors are αβ-heterodimers, of which two main isoforms, α₁β₁ and α₂β₂, exist at differing levels in different tissues (5). Studies on purified receptors (6) and intact cells (7) suggest that activation of GC occurs very rapidly, within a few ms or less of adding NO, and complete deactivation in cells (upon removal of NO) requires only about 500 ms (7). Furthermore, when studied in isolation, the receptors are highly sensitive NO detectors, with only about 1 nM being needed to evoke half-maximal GC activity (8). The shape of the resulting cGMP response in cells, however, varies greatly from one cell to another. For example, in platelets (9, 10), mast cells (11), and a neuroblastoma cell line (12), the response to continued NO application is a transient increase in cGMP, whereas, in a population of glial cells from the cerebellum, cGMP rises hyperbolically with time to attain large plateau concentrations (10). An intermediate response is found in neurons in another part of the brain (13) and in aortic smooth muscle (14). The information encoded in the different cGMP profiles is unclear, but the temporal and amplitude characteristics presumably impact on the selection of downstream targets, which include protein kinases in the nanomolar concentration range (15) and ion channels in the micromolar range (16).

The aim of the present study was to analyze quantitatively the factors responsible for shaping cellular cGMP signals in response to NO and to determine the relationship between cGMP signals and subsequent protein phosphorylation. The levels of cGMP in a cell reflect the interplay between the rate of synthesis, the rate of degradation by phosphodiesterase (PDE), and, possibly, the rate of extrusion into the extracellular fluid. The rates of cGMP synthesis and degradation may both change with time because of desensitization of the GC-coupled NO receptors (10) and enhancement of PDE activity (17, 18), rendering the problem a complex one. To address it, we have chosen the simplest cells available, blood platelets. Platelets are very small (~1 μm in diameter), which diminishes problems of subcellular compartmentalization (19) and minimizes the diffusion distance from the suspending medium to the cell interior. They are also homogenous and, being an established target for NO, are rich in GC-coupled NO receptors, PDE (specifically a cGMP-prefering isofrom, PDE5), and vasodilator-stimulated phosphoprotein (VASP), a substrate for cGMP-dependent protein kinase (20).

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1 The abbreviations used are: NO, nitric oxide; GC, guanylyl cyclase; PDE, phosphodiesterase; VASP, vasodilator-stimulated phosphoprotein; SPER/NO, spermine/NO adduct; CPTIO, 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; ODQ, 1-H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.
EXPERIMENTAL PROCEDURES

Preparation of Platelets—Whole blood was collected from adult Sprague-Dawley rats (3–5 rats/experiment) into acid citrate dextrose solution (12.5%) and centrifuged at 300 × g for 10 min at 20 °C. The platelet-rich plasma was withdrawn and recentrifuged to eliminate residual red and white blood cells. The supernatant was centrifuged for 10 min at 2000 × g (12 °C), and the platelet pellet was resuspended in buffer containing 137 mM NaCl, 0.5 mM MgCl₂, 0.55 mM Na₂HPO₄, 2.7 mM KCl, 25 mM HEPES, and 5.6 mM d-glucose, pH 7.4, at 37 °C, to give a final concentration of 0.5 mg protein/ml (protein being measured using the bicinchoninic acid method). The platelet suspension was incubated in 1–2 ml volumes for at least 1 h at 37 °C prior to use. t-1-nitroarginine (100 μM) was included at the start of the incubation to abolish possible complications arising from endogenous NO synthesis.

Delivery of NO—The platelets were exposed to clamped NO concentrations using a new method (8). Briefly, spermene/NO adduct (SPER/NO) was used as the source of NO and 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO; 200 μM) was included as an NO sink, serving to translate rapidly (within 1 s) different rates of NO release from the donor into proportionate steady-state concentrations. Urate (300 μM) was added to convert NOₓ to NO, and superoxide dismutase (1000 units/ml) was included as a precautionary measure to remove any superoxide ions that would otherwise react with NO. The method had previously been used only in cell-free preparations, so it was important to evaluate its use with living cells. In accordance with predictions (see Supplemental Data, Fig. S1), the addition of SPER/NO in increasing concentration (100 μM to 1 mM) to a platelet suspension (0.5 mg protein/ml) containing CPTIO, urate, and superoxide dismutase (at 37 °C) resulted in increasing steady-state NO concentrations, as measured using an electrochemical probe (Iso-NO; World Precision Instruments, Stevenage, Herts, UK). A plot of the amplitude of the plateau NO concentration against SPER/NO concentration gave the expected straight line passing through zero and the slope (1.3 × 10⁻⁸) was similar to that found beforehand in cell-free preparations (8). Omission of platelets had no effect on the NO concentration profile, indicating that they do not consume NO significantly themselves. These data validate the method for delivering NO in the 0–100 μM concentration range to platelet suspensions. The NO concentration generated by the addition of 400 μM SPER/NO to the platelet buffer (containing SPER/NO, CPTIO, urate, and superoxide dismutase) was measured 3–4 times on each experimental day and averaged for the purpose of calibration.

Measurement of cGMP—The method was similar to that used previously (10). In short, aliquots of the platelet suspension (usually 50 μl) were withdrawn before and at various times after the addition of SPER/NO to give different NO concentrations and expelled immediately into inactivation buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.4) at 0 °C. The aliquots were maintained at that temperature for about 10 min. When used, 1-H-[1, 2, 4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a blocker of GC-coupled NO receptors (21), was added to the platelets 15 min before the addition of SPER/NO in order to achieve a steady degree of inhibition (22). The levels of cGMP were measured by radioimmunoassay and expressed relative to the amount of protein. To measure extracellular cGMP, aliquots of the suspension were filtered gently but rapidly (5 s) through 0.22-μm cut-off filters. Usually, three independent runs were carried out in each experiment, and the resulting data are presented as means ± S.E.

Measurement of GC Activity in Lysed Platelets—Platelets were prepared and incubated as described above. An equal volume of 5-fold concentrated lysis buffer containing 300 mM diethytothreitol and two Mini Complete EDTA-free protease inhibitor tablets per 10 ml (Roche Applied Science) was added to platelet suspensions, and the mixture was sonicated and kept on ice. Lysed platelets were assayed in 50 mM Tris-HCl buffer (pH 7.4) supplemented with 3 mM MgCl₂, 0.1 mM EGTA, 0.05% (w/v) bovine serum albumin, 1 mM 3-isobutyl-1-methylxanthine, 5 mM creatine phosphate, 200 μg/ml creatine phosphokinase, 300 mM GTP, 1000 units/ml superoxide dismutase, 300 μM uric acid, and 200 mM CPTIO. SPER/NO was added, and 100-μl aliquots were removed at various intervals, inactivated, and assayed for cGMP as for the intact platelets. Upon the addition of 2 or 20 μM NO, cGMP accumulated linearily with time for at least 2 min (not illustrated) as reported previously for cell-free preparations (8); a 2-min exposure was used to obtain concentration-response data.

Analysis of Rates of cGMP Synthesis and Degradation—The kinetic parameters of the PDE activity were measured by exposing platelets to NO in the presence of varying concentrations of a PDE5 inhibitor (sildenafil), and then the decay in cGMP was followed after removing NO using hemoglobin. The data were analyzed using the linear transformation of the integrated Michaelis-Menten equation (23),

\[
\frac{t}{\ln \left(\frac{P_0}{P_t}\right)} = \frac{1}{V_p} \ln \left(\frac{P_0}{P_f}\right) + \frac{K_p}{V_p}
\]

(Eq. 1)

where \(V_p\) represents the limiting PDE activity, \(K_p\) is the apparent Michaelis-Menten constant, and \(P_0\) and \(P_t\) are the cGMP levels at the start and at time \(t\), respectively. For a competitive inhibitor such as sildenafil, when used in concentrations that are large relative to the inhibitory constant \(K_i\), the value of \(K_v/V_p\) (the intercept) should increase in proportion to the inhibitor concentration ([I]) because of the following relationship:

\[
K_v = K_p \left(1 + [I]/K_i\right)
\]

Linear regression analysis of the transformed cGMP decay was used to obtain best estimates of the gradient (1/\(V_p\)) and of a common intercept per unit concentration of sildenafil. From this intercept and the value of \(V_p\), the ratio \(K_v/K_p\) was obtained.

To analyze the cGMP profiles formed in response to NO, the basic premise was that the level of cGMP at any time is governed by the rates of synthesis (\(v_s\)) and degradation (\(v_d\)), since the rate of cGMP extrusion from the cells was negligible under most conditions (see “Results”). The methods for determining the parameters under conditions of maximal PDE inhibition (100 μM sildenafil), when the cGMP accumulation curve followed a hyperbolic shape, were as before (10). Briefly, the curve was fit (in Origin 7, OriginLab Corp., Northampton, MA) to a generalized hyperbola of the form:

\[
P = \frac{a v_s e^{t/v_d}}{K_v + a v_s e^{t/v_d}}
\]

(Eq. 2)

where \(P\) represents the cGMP level, \(t\) is time, \(a\) is maximum cGMP accumulation, and \(v_s\) and \(v_d\) are constants. Differentiating and inserting the result into the expression \(dP/dt = v_s - v_d\), gives the following:

\[
\frac{a^n h^e^{-t/v_d}}{[k_e + v_d]}v_s - v_d = \frac{a^n h^e^{-t/v_d}}{[k_e + v_d]}v_s
\]

(Eq. 3)

from which \(v_s\) was found with respect to \(t\).

In some cases, cGMP did not reach a steady plateau but fell gradually after reaching a peak despite PDE being substantially inhibited (100 μM sildenafil). In these cases, the curves were fit (in Origin) to a pulse function,

\[
P = \frac{A(1 - e^{-(b/t)}))(e^{-(b/t)})}{e^{-(b/t)} - e^{-(b/t)} - e^{-(b/t)} - e^{-(b/t)}}
\]

(Eq. 4)

where \(A\) dictates the maximum cGMP level, \(t_s\) and \(t_f\) are the time constants for the rising and falling phases, and \(b\) is a constant. Upon differentiation, this equation gives the following:

\[
\frac{dP}{dt} = \frac{A(1 - e^{-(b/t)}))(e^{-(b/t)} + e^{-(b/t)} + e^{-(b/t)} + e^{-(b/t)})}{e^{-(b/t)} - e^{-(b/t)} - e^{-(b/t)} - e^{-(b/t)}}
\]

(Eq. 5)

which is used to find \(v_s\) as above.

A more complex model was developed to analyze the responses under conditions where PDE was not inhibited or was inhibited more mildly. The rate of cGMP synthesis \(v_s\) was represented by the following equation,

\[
v_s = \frac{GC_{\text{max}} - c[\text{NO}]}{EC_{\text{max}} + [\text{NO}]} e^{-k_t t} + c + (1 - e^{-k_t t})
\]

(Eq. 6)

where \(GC_{\text{max}}\) is the limiting GC activity, \(k_t\) is the desensitization rate constant, \(c\) is a nondesensitizing component, and \(k_t\) is the rate constant for its activation. Because of mixing delays and the fact that it takes a finite time (about 1 s) for the NO concentration to reach steady state (8), the NO concentration in the above equation was assumed to increase exponentially with a rate constant of 1.5 s⁻¹ (giving 90% of the steady-state level in 1 s), and this was also the value assigned to \(k_t\). To enable cGMP levels to rise before desensitization sets in (see “Results”), it was assumed that \(k_t\) increases from zero (at \(t = 0\)) to its final value with a rate constant of 0.8 s⁻¹. The EC₅₀ for NO was taken as 10 nM (see “Results”), and the value of \(GC_{\text{max}}\) was adjusted according to the amplitude of the maximum cGMP response in any particular experiment, which could vary by up to 2-fold.

Analysis of the PDE activity \(v_d\) was based on the Michaelis-Menten equation, modified for the presence of a competitive antagonist (as needed).
PDE5 activity is not fixed, but it becomes enhanced when cGMP binds to the enzyme (17). This enhancement corresponds to a time-dependent increase in the limiting rate of hydrolysis ($V_L$), a reduction in the $K_m$ for cGMP, and an increase in the affinity of sildenafil ($K_i$). Accordingly, these parameters were represented as follows,

$$
V = \frac{V_L [\text{cGMP}]}{K_a (1 + [I]/K_i) + [\text{cGMP}]}
$$

where $V_a$ is the basal condition, and $V_p$ is the component that enhances with rate constant $k_p$,

$$
K_a = K_{a_2} + K_{a_1}(e^{-k_p t})
$$

where $K_{a_2}$ is the final value that is approached with rate constant $k_p$, from the initial value ($K_{a_2} + K_{a_1}$).

$$
K_i = K_{i_2} + K_{i_1}(e^{-k_p t})
$$

where $K_{i_2}$ is the final value that is approached with rate constant $k_p$, from the initial value ($K_{i_2} + K_{i_1}$).

The equations assume that the enhancement is irreversible, which, considering the short time intervals being analyzed, is justified (17, 18). With values assigned to the variables, the cGMP profile is obtained by numerically solving the following equation.

$$
\frac{dP}{dt} = v_a - v_d
$$

The equations were solved using the adaptive Runge-Kutta algorithm in Mathematica, version 2000ii 11 (Adept Scientific, Letchworth, Herts, UK).

**Measurement of VASP Phosphorylation**—Platelets were incubated and exposed to clamped NO concentrations as above. Aliquots (100 µl) were removed at specified time points and inactivated in 33 µl of 4-fold concentrated SDS-loading buffer, containing 250 mM Tris-HCl (pH 6.8), 8% (w/v) SDS, 0.04% (w/v) brom phenol blue, 40% (v/v) glycerol, and 2% (v/v) β-mercaptoethanol. Concentration-response experiments used 1-min exposures to NO. Samples were heated to 100°C for 5 min, and then aliquots containing 5 µg of protein were separated by electrophoresis on a 10% SDS-PAGE gel. Proteins were then transferred to nitrocellulose membrane (Hybond ECL; Amersham Biosciences) and blocked for 2 h in 3% (w/v) bovine serum albumin in buffer containing 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% (v/v) Tween 20. Membranes were then probed with an anti-phospho-VASP antibody (0.05 µg/ml; Alexis, USA) and anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1.67 µg/ml; Chemicon, Hampshire, UK) before being probed with anti-mouse-horseradish peroxidase antibody (1:10,000; DAKO, Ely, Cambridgeshire, UK). Protein bands were visualized using an enhanced chemiluminescence kit (ECL Plus; Amersham Biosciences). Densitometry was carried out using Optiquant (PerkinElmer Life Sciences), and differences were analyzed statistically using the two-tailed Student’s t test.

**Reverse Transcriptase-PCR**—TRIZol reagent (Invitrogen) was used to isolate total RNA from the platelet suspensions according to the manufacturer’s instructions. First strand cDNA synthesis was performed using Thermostart reverse transcriptase (Invitrogen). Platinum TaqDNA polymerase (Invitrogen) was subsequently used to generate PCR products. The primers and annealing temperatures used are listed in Table I. The reaction conditions were as follows: 94°C for 5 min, annealing temperature for 2 min, and 72°C for 1 min followed by 40 cycles of 94°C for 30 s, annealing temperature for 30 s, 72°C for 30 s, and a final incubation at 72°C for 5 min. Reaction products were detected by standard agarose gel electrophoresis.

**RESULTS**

**Expression of GC-coupled NO Receptor Subunit mRNA in Rat Platelets**—Although anucleate, platelets contain abundant mRNAs that can be translated into proteins (24). Reverse transcriptase-PCR products of the mRNAs encoding both the α₁ and β₁ subunits were detected, whereas the products of α₂ and β₂ mRNAs were not (Fig. 1). Thus, rat platelets appear to express only the α₁β₁ NO receptor, as found in other species (25). The result also indicates that the expression of the receptor protein in platelets could be subject to regulation (24).

**Potency of NO for Platelet GC-coupled Receptors**—The addition of fixed NO concentrations to platelet suspensions led to very transient increases in cGMP (Fig. 2a). At the higher NO concentrations (10–50 nM), the peak elevation occurred after 5 s, but, as the concentration was reduced, the peak gradually shifted to 2 s (2 nM NO). The NO concentration range active in the intact platelets appears higher than in cell-free preparations, where maximal effects are observed at about 10 nM NO (8). To investigate whether the platelet receptor is unusual, aliquots of the same batch were either lysed or kept intact and compared directly. In lysed platelets, the EC₅₀ for NO was 1.7 nM, and the Hill coefficient was near 1. In intact platelets given a short (5-s) exposure to NO in the presence of the PDE5 inhibitor sildenafil (100 µM; see below) the EC₅₀ value was 11 nM, with the Hill coefficient remaining close to 1 (Fig. 2b).

**Kinetics of cGMP Degradation**—Human platelets contain PDE5 as the main cGMP-hydrolyzing enzyme, although PDE2 can make a minor contribution (10, 14). With the PDE5 inhibitor sildenafil (1 µM) present, the NO-evoked cGMP response was transformed from a brief transient to a larger, broader transient followed by a prolonged plateau (Fig. 3a). The main effect of increasing the sildenafil concentration further (3–100 µM) was to increase the amplitude of the plateau. Ultimately, the response had a hyperbolic shape, with the maximum amplitude being reached after about 20 s. The PDE2 inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (100 µM) on its own had no effect on the response to NO (50 nM) measured after a 60-s exposure, and, when added together with 100 µM sildenafil, it did not raise cGMP further (results not shown), suggesting that PDE2 makes a negligible contribution to cGMP hydrolysis in rat platelets.

Knowledge of the PDE kinetics is a prerequisite for analyzing cellular cGMP profiles. To extract this information, platelets were stimulated with 50 nM NO in the presence of a range of sildenafil concentrations (1–100 µM) for a period of 1 min, by which time cGMP levels were close to steady-state (Fig. 3a). Hemoglobin (30 µM) was then added to remove NO, and the decline in cGMP was followed for 3 min (Fig. 3b). There was no observable delay between the addition of hemoglobin and the fall in cGMP levels, consistent with GC deactivating in the subsecond time frame (7), and the rates of decline varied inversely with the sildenafil concentration. Analysis of the data
Kinetics of NO Signal Transduction

FIG. 1. Identification of GC-coupled NO receptor subunits expressed in rat platelets. The bands show reverse transcriptase-PCR products of the negative control (distilled water, –), positive control (plasmid containing the subunit, +), and platelet mRNA (pl) for the α1, β1, α2, and β2 subunits.

FIG. 2. Concentration-cGMP response curves for NO in rat platelets. a, time course of cGMP accumulation in intact platelets (no sildenafil) in response to 2–50 nM NO. b, comparison of the sensitivity of GC-coupled receptors to NO in lysed and intact platelets (left and right abscissae, respectively), the data being fit by the Hill equation. For intact platelets, the exposure was for 5 s in the presence of 100 μM sildenafil. Data are means ± S.E. (n = 3).

using the linear transformation of the integrated Michaelis-Menten equation (Fig. 3c) (see “Experimental Procedures”) gave a value of the limiting rate of cGMP hydrolysis (Vp) of 250 pmol/mg protein/s. For platelets, 1 mg of protein corresponds to 2.5 μl (26), so that, assuming homogeneity, the value of Vp corresponds to about 100 μM/s in this experiment. From the intercepts and the value of Vp, the ratio Km/Ki is 400. Taking the steady-state Kp of PDE5 for cGMP to be 1 μM (17), the Ki for sildenafil is 2.5 nM, which accords with published values (27). Simulations using the derived parameters tracked the measured cGMP decays at each sildenafil concentration (Fig. 3b). Subsequent analyses used the same Kp and Ki values, but the precise Vp value (along with the value for the maximal GC activity) had to be adjusted for each experiment, because the absolute cGMP levels varied. The values fitting all of the data in the present study ranged from 150 to 220 pmol/mg protein/s (equivalent to 67–100 μM/s). The corresponding range of limiting GC activities (see below) was 237–360 pmol/mg protein/s (95–144 μM/s).

cGMP Extrusion—When platelets were stimulated with 50 nM NO for 1 min in the presence of 100 μM sildenafil, the total cGMP was 1345 ± 31 pmol/mg protein, of which extracellular cGMP accounted for 35 ± 1 pmol/mg protein (n = 3). This small amount (about 3% of total) is within the measurement error and therefore can be ignored. Extracellular cGMP during the transient response in the absence of sildenafil was also negligible (<2% of the peak), but its persistence meant that it constituted about half of the total after 1 min, the total then being about 10 pmol/mg protein. In the presence of 1 μM sildenafil, cGMP efflux occurred in two linear phases, the first being about twice the rate of the second. The rates were only low (0.5–2 pmol/mg protein/s), so extracellular cGMP again comprised a negligible component except after long exposures (e.g. it constituted 20% of the total after 2 min; see Supplemental Data, Fig. S2).

NO-evoked GC Activity with PDE Inhibited—To start to unravel the GC kinetics at different NO concentrations, the initial experiments were carried out with minimal PDE activity (100 μM sildenafil). At all NO concentrations tested (1–50 nM), cGMP accumulated hyperbolically (Fig. 4a). The initial slopes increased with NO concentration, and, at 3 nM NO and above, the same maximum accumulation was ultimately achieved. At 1 nM NO, cGMP settled at about 60% of maximum after 2 min. Since the PDE activity is so low, the curving-off with time represents GC desensitization (10). Analysis of the GC activity profiles (see “Experimental Procedures”) indicated that the peak occurred within 2–6 s and increased more than 10-fold as NO rose from 1 to 50 nM (Fig. 4b). Fitting the peaks to the Hill equation indicated an EC50 of 9 nM, a value very similar to that determined from 5-s exposures to NO (Fig. 2b) and a limiting rate of 280 pmol/mg protein/s (~110 μM/s). After the peaks, desensitization occurred at differing rates. At 50 nM NO, there was almost complete desensitization within 20 s, whereas at 1 nM NO, there was some residual activity (about 20% of the peak) after 2 min. GC desensitization in other cells occurs exponentially (7), and, in platelets, the derived falling phases of
GC activity could likewise be fit with single exponential functions (not illustrated), allowing desensitization rate constants to be extracted. A plot of the rate constants against the peak GC activities indicated an approximately linear relationship (Fig. 4c).

The faster desensitization rates observed as the NO concentration was raised could be explained by the phenomenon being either NO-dependent or GC/cGMP-dependent. To distinguish between these alternatives, the NO concentration was kept constant (50 nM) and GC activity varied using a range of concentrations (not illustrated), allowing desensitization rate constants, obtained by fitting single exponentials to the falling phases of the GC activity (from 5 s onward), against the peak GC activity, both from b.

GC and PDE Activities without PDE Inhibition—This more complex situation was approached by periodically adding sildenafil (100 μM) to NO-stimulated platelets. By rapidly removing PDE activity, this method should allow the underlying GC activity to be revealed (14, 19). Adding sildenafil immediately (∼1 s) before the NO (50 nM) resulted in the usual large plateau (Fig. 5a), signifying rapid penetration of the inhibitor. When sildenafil was added after 5-, 10-, and 60-s exposure to NO, the initial rate of cGMP accumulation progressively slowed. There was also a gradual reduction in the maximum level achieved such that, by 60 s, the steady-state cGMP amplitude was about 65% of control. Deconvoluting these progress curves indicated that the peak GC activity fell to 22% of its starting value (Fig. 5b) with a rate constant of 0.25 s⁻¹ (Fig. 5c). The rate constant was the same as that measured from the decline in GC activity with sildenafil present at the beginning (0.26 s⁻¹) (Fig. 5b). In this latter situation, however, desensitization was more complete, being equivalent to a 99% loss of peak amplitude compared with 80%.

With sildenafil present at the start, there is the simple situation of very low PDE activity throughout, so the cGMP response is determined almost entirely by the GC activity profile (Fig. 5d). To unscramble the contributions of GC and PDE activities to the transient response occurring in the absence of sildenafil, GC activity was modeled on the Hill equation, assuming that the EC₅₀ for NO is 10 nM (see above) and that it comprises desensitizing and nondesensitizing components (see "Experimental Procedures"). When the experimentally measured variables were inserted into the model (see Fig. 5 legend), the simulated profile of cGMP synthesis resembled the profile deduced experimentally (Fig. 5, a and c).

PDE5 activity is not fixed but becomes enhanced when cGMP binds to the enzyme (17). This enhancement corresponds to a time-dependent increase in the limiting rate of hydrolysis (Vₑ), a reduction in the Kₑₐₚₜ for cGMP, and an increase in the affinity of sildenafil (Kₛ). From literature values for recombinant PDE5 (17) and the steady-state kinetic parameters governing PDE activity in intact platelets (see above), it was assumed that the Kₑₐₚₜ falls from 5 to 1 μM and the Kₛ falls from 5 to 2.5 nM; the changes in Vₑ were deduced from fits to the experimental results. Inspection of the data suggests that the starting value of Vₑ must be low because 100 μM sildenafil has little influence on the cGMP response to 50 or 10 nM NO at a 2-s time point, but it must increase quite rapidly, because, by 5 s, sildenafil has a large effect (see Figs. 3a–6a). A good fit to the results of this experiment and all others was obtained by assuming a basal Vₑ, which becomes enhanced 7-fold with time. At the higher NO concentrations (see below), all of the data could be accommodated by assigning a rate constant for PDE enhancement of 0.15 s⁻¹ (fits were worse if it was lowered to 0.1 s⁻¹ or raised to 0.2 s⁻¹; not shown).

The model accurately simulated the experimental data (Fig. 5e). The shape of the cGMP response is thereby explained by PDE activity at the start being low and GC activity being high, allowing cGMP to build up. The combination of GC desensitization and PDE enhancement then results in a slowing of the rate of cGMP accumulation until, at 5 s, the two become equal (corresponding to the cGMP peak). Continued PDE enhancement and GC desensitization causes cGMP levels to fall. Once the cGMP levels are down to within the range of the Kₑₐₚₜ (1 μM, or about 3 pmol/mg protein), PDE activity (being substrate-linked) becomes rapidly reduced, and, thereafter, cGMP remains at a low level and is consumed at the rate at which it is synthesized.

Similar experiments probed the kinetics with NO concentrations giving less than maximal cGMP responses. At 10 nM NO, GC still desensitized (Fig. 6, a–c), but the rate (kd = 0.072 s⁻¹) was about 3-fold slower than at 50 nM NO. It was also lower than the desensitization rate at 10 nM NO when sildenafil was added at the beginning (0.2 s⁻¹ in this experiment; see also Fig. 4). The final GC activity, however, was the same as was found at the higher NO concentration (23% of the maximum). In addition, the maximum cGMP accumulation upon the addition...
of sildenafil also became progressively lowered (Fig. 6a) to a similar extent (62% of control) to that found at the higher NO concentration. Simply by inserting the values for the NO concentration and the measured desensitization rate constants and keeping all other variables the same, the model accurately simulated the cGMP responses at both NO concentrations (Fig. 6, a and d). Hence, combining the lower GC activity and GC desensitization rate with identical PDE parameters is sufficient to account for the response to 10 nM NO having a 50% desensitization. The maximum amplitude after the addition of sildenafil (100 μM) was added ~1 s before (open squares) and then 5 s (filled circles), 10 s (open circles), and 60 s (triangles) after the addition of NO. Data are means ± S.E. (n = 3), and the sildenafil-induced cGMP elevations were fitted by generalized hyperbolae. GC activities (vₐ) corresponding to the data in a; curves are labeled with the times of sildenafil addition (s). c, plot of the peak amplitude of GC activity from b against the time of the addition of sildenafil; the data are fitted to a single exponential, giving a rate constant of 0.25 s⁻¹. d, simulated profiles of the rates of cGMP synthesis (vₛ, dashed line) and degradation (vₕ, dotted line) underlying the cGMP response (solid line) with sildenafil added at the beginning. The simulated response is overlaid on the experimental data in a (line). e, the simulated profiles of the rates of cGMP synthesis (vₛ, dashed line) and degradation (vₕ, dotted line) giving rise to the cGMP response (solid line, also shown in a) in the absence of sildenafil. The kinetic parameters used in the model (see “Experimental Procedures”) were as follows: Vₚₛ = 150, GCₚₘₐₓ = 237, and c = 37 (all in pmol/mg protein/s), kₛ = 0.25 s⁻¹, and kₕ = 0.15 s⁻¹.

This experiment also provided an opportunity to test an aspect of the model that is not verifiable, at least qualitatively, by inspection of the data. At the plateau, the GC and PDE activities are predicted to be the same and equal to 52 pmol/mg protein/s in this experiment. To test this prediction, the GC activity was exposed by adding a high sildenafil concentration (100 μM), and the underlying PDE activity was revealed by the addition of hemoglobin (30 μM). The initial GC activity was 57 pmol/mg protein/s (Fig. 8b), and the decay in cGMP upon the addition of hemoglobin was accurately predicted by the steady-state PDE parameters, giving an initial rate of hydrolysis of 50 pmol/mg protein/s (Fig. 8a).

Since the measured enzyme activities underlying the cGMP response at steady-state were very close to expectations, the same analysis was applied to an earlier experiment carried out primarily to determine the PDE activity at different sildenafil concentrations (Fig. 3, a–c). At the resulting plateau cGMP concentrations, it can be assumed that the initial rates of cGMP degradation and synthesis are equal. From the measured degradation rates, the GC activity remained constant at about 65 pmol/mg protein/s when the steady-state cGMP levels were up to 50% of the maximum (Fig. 3d). The maximum amplitude in this experiment would require a peak GC activity of about 300 pmol/mg protein/s, so the steady-state GC activity over this range represents 80% desensitization, as found repeatedly in other experiments. Above 50% of the maximum cGMP, the steady-state GC activity fell sharply to reach less than 5 pmol/mg protein/s (98% desensitization) at the highest sildenafil concentration (Fig. 4d). The midpoint of the addi-
tional desensitization occurred at 70% of the maximum.

Kinetics of VASP Phosphorylation—Phosphorylation of the protein VASP occurs in platelets in response to activity of the NO-cGMP pathway. The initial phosphorylation at serine 239 (hereafter called VASP-1P) depends on cGMP-dependent protein kinase, whereas a second phosphorylation at serine 157 (VASP-2P) can occur through cGMP- or cAMP-dependent kinase, whereupon a second phosphorylation at serine 157 (hereafter called VASP-1P) depends on cGMP-dependent protein kinase in both VASP-1P and VASP-2P at higher NO concentrations occurring despite increased cGMP levels, suggesting that it results from a cGMP-independent inhibitory effect of NO.

At concentrations higher than 3 nM, the level of VASP-1P declined. VASP-2P required higher NO concentrations than VASP-1P, being significant at 3 nM ($p < 0.001$) and peaking at 3–30 nM NO before also declining (Fig. 9, d and e). The decline in both VASP-1P and VASP-2P at higher NO concentrations occurred despite increased cGMP levels, suggesting that it results from a cGMP-independent inhibitory effect of NO.

**DISCUSSION**

The results address several outstanding questions concerning cellular NO-cGMP signaling: the potency of NO for its receptors, the temporal changes in GC and PDE activities, the ways that cGMP synthesis and degradation interact to shape cGMP responses, and the relationship between NO-evoked cGMP increases and downstream protein phosphorylation. There was previously little or no knowledge of any of these basic issues, mainly because it had not been possible to supply NO in the controlled manner that is mandatory for obtaining interpretable data. The successful application of the new NO delivery method to intact platelets opens the way for investigating effects of NO on living cells under conditions that would be axiomatic for other signaling molecules.

**Differential Potency of NO for GC-coupled Receptors in Cells and Lysates**—The potency of NO for evoking GC activity in the platelets ($EC_{50} = 10$ nM) was about an order of magnitude lower than in platelet lysates. The value in lysates is close to that found for purified lung receptor (predominantly also the $\alpha_1 \beta_2$ isoform) and for lysates of cells expressing the $\alpha_1 \beta_1$ receptor ($8$), suggesting that the lower $EC_{50}$ in the cells is the peculiarity. A plausible explanation is that the affinity of the
receptors for NO differs in the two conditions. In cells, deactivation of GC upon the removal of NO was found to occur with a half-time of 0.2 s (7), which is about 10-fold faster than reported for the purified protein (29, 30). Assuming that the deactivation rates reflect the rates of dissociation of NO from its receptors and that the association rates are the same, the receptor in cells would have a 10-fold lower affinity for NO, thereby accounting for the potency shift. As to a possible mechanism, there is a site on the receptor protein whose pharmacological occupation leads to a reduction in the rate of deactivation and a corresponding increase in the potency of NO (29, 30), so it is conceivable that there is an endogenous agonist for this site that does the reverse.

Desensitization of GC-coupled NO Receptors—GC desensitization had been described for cerebellar astrocytes, which naturally have only very low PDE activity (10), and for other cells when PDE activity was artificially reduced (10, 13). The relevance of desensitization to cells that have higher PDE activity and/or to NO concentrations giving less than maximal stimulation was not known. The results suggest that the kinetics of GC desensitization is instrumental in shaping cGMP responses to NO over most of its active concentrations and over the entire spectrum of PDE activities. Apparently in partial disagreement with this conclusion is a previous report on normal human platelets (no PDE inhibitor) stating that GC activity did not change during prolonged exposure to NO (14), whereas, in our experiments, there was prominent GC desensitization except at low NO concentrations. Whereas there are several methodological differences, including the way that the NO was delivered and the source of the platelets, the previous inference was based on manipulated data from an intermediate 6-s segment of the rise in cGMP following PDE inhibition. To draw valid conclusions, it is essential to follow the whole trajectory.

Both with normal PDE activity and with a reduction in PDE activity by up to 100-fold (i.e. with up to 3 μM sildenafil), 80% of the maximum NO-evoked GC activity was subject to desensitization. This meant that, at an NO concentration giving less than 20% of the maximum, GC was non-desensitizing, and, at higher NO concentrations, it desensitized down to this level.
The residual 20% of GC activity became desensitized only at the higher sildenafil concentrations, suggesting that this component is relevant only to cells that naturally have low PDE activity, such as cerebellar astrocytes (10). The mechanism responsible for GC desensitization is unknown and is currently under investigation. Previously, circumstantial evidence indicated that it was related to the level of cGMP (13), and all of the data in the present study (with and without PDE inhibition) would be consistent with this proposal.

In addition to desensitization (measured from the initial rate of GC activity), there was also a time-dependent fall in the maximum capacity for cGMP accumulation, amounting to about 35% over 1 min. This reduction is not obviously related to the enhancement, because the cGMP added to monitor catalysis also augments the activity (17). The enhancement involves cGMP binding to a high affinity GAF domain of the protein (14). The assay method is likely to underestimate the extent of the enhancement, because a significant proportion of the cGMP will be bound to kinases and other cGMP receptors, rendering it unavailable to the PDE (34). Hence, the rate and extent of PDE5 activity increased at a fixed rate (half-time of about 5 s), which is consistent with an early saturation of the GAF domain by the high initial rate of cGMP synthesis (50–100 µM/s). Despite being constant, the time course of the enhancement provided the framework whereupon variations in GC activity and associated desensitization rate functioned to sculpt the final cGMP response. Changes in the kinetics of PDE enhancement are more likely to occur at lower NO concentrations, as suggested by the fact that the rate constant had to be reduced 3-fold to account for the shape of the cGMP response to 3 nM NO. Analysis of such small cGMP responses is problematic, however, because a significant proportion of the cGMP will be bound to kinases and other cGMP receptors, rendering it unavailable to the PDE (34). Hence, the rate and extent of PDE5 enhancement in cells at low NO concentrations remain uncertain.

Application to Other Cells—The limiting NO-evoked GC activity in the platelets corresponds to about 100 µM cGMP being formed each second. Although this rate seems high, platelets are not unique in this respect, because, after making corrections for the purity of the cells in question, an activity of this order exists in astrocytes from the cerebellum (10) and in a
The rise in cGMP, indicating activation of cGMP-dependent downstream pathways. Whatever the NO profiles might be, the results begin to allow predictions of how they would be transduced into cGMP signals in different target cells and hence promote downstream phosphorylation.

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REFERENCES

1. Colquhoun, D., and Sakmann, B. (1998) Neuron 20, 381–387
2. Beridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Nat. Rev. Mol. Cell. Biol. 4, 517–529
3. Hobbs, A. J., Higgs, A., and Moncada, S. (1999) Annu. Rev. Pharmacol. Toxicol. 39, 191–220
4. Garralda, J., and Boulton, C. L. (1995) Annu. Rev. Physiol. 57, 683–706
5. Friese, A., and Koelsch, D. (2003) Circ. Res. 93, 96–105
6. Zhao, Y., Brandish, P. E., Ballow, D. P., and Marietta, M. A. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 14753–14758
7. Bellamy, T. C., and Garralda, J. (2001) J. Biol. Chem. 276, 4287–4292
8. Griffiths, C., Wykes, V., Bellamy, T. C., and Garralda, J. (2003) Mol. Pharmacol. 64, 1349–1355
9. Mellon, B. T., Ignarro, L. J., Ohlistein, E. H., Pontecorvo, E. G., Hyman, A. L., and Kadowitz, P. J. (1981) Blood 57, 946–955
10. Bellamy, T. C., Wood, J., Godwin, D. A., and Garralda, J. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 2526–2530
11. Bidri, M., Becherel, P. A., Le Goff, L., Pieroni, L., Guillossin, J. J., Debre, P., and Aruck, M. (1995) Biochim. Biophys. Acta 1250, 507–517
12. Schlabach, T., and Deeg, T. (1997) Biochim. Biophys. Acta 136, 473–480
13. Wykes, V., Bellamy, T. C., and Garralda, J. (2002) J. Neurochem. 83, 37–47
14. Mullershausen, F., Rusuwurm, M., Thompson, W. J., Liu, L., Koelsch, D., and Friese, A. (2001) J. Cell Biol. 155, 271–278
15. Francis, S. H., and Corbin, J. D. (1999) Crit. Rev. Clin. Lab. Sci. 36, 275–328
16. Kaupp, U. B., and Seifert, R. (2002) Physiol. Rev. 82, 759–824
17. Rybalkin, S. D., Rybalkina, I. G., Shimizu-Alberge, M., Tang, X. B., and Beavo, J. A. (2003) EMBO J. 22, 469–478
18. Mullershausen, F., Friese, A., Feil, K., Thompson, W. J., Hofmann, F., and Koelsch, D. (2003) J. Cell Biol. 160, 719–727
19. Bellamy, T. C., Pagan, K. A., Tue, T. E., Schaack, J., Cooper, D. M., and Karpen, J. W. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 13049–13054
20. Schwarz, U. R., Walter, U., and Eigenhalter, M. (2001) Biochem. Pharmacol. 62, 217–228
21. Garralda, J., Southam, E., Boulton, C. L., Nielsen, E. B., Schmidt, K., and Eigenthaler, M. (2001) Eur. J. Biochem. 257, 471–481
22. Moreland, R. B., Goldstein, I., Kim, N. N., and Traish, A. (1999) Trends Endocrinol. Metab. 10, 97–104
23. Lipshutz, A., Ishii, C., Reinhard, K., Honig-Liedl, P., Jarchau, T., Hoschuetzky, H., and Walter, U. (1999) J. Biol. Chem. 274, 20029–20035
24. Rusuwurm, M., Mergia, E., Mullershausen, F., and Koelsch, D. (2002) J. Biol. Chem. 277, 24883–24888
25. Schmidt, P., Schramm, M., Schroder, H., and Stasch, J. P. (2003) Eur. J. Pharmacol. 488, 184–188
26. Bellamy, T. C., and Garralda, J. (2002) Br. J. Pharmacol. 136, 95–103
27. Cornish-Bowden, A. (1995) Fundamentals of Enzyme Kinetics, Portland Press, London
28. Lindemann, S., Tolley, N. D., Dixon, D. A., McIntyre, T. M., Prescott, S. M., and Zimmerman, G. A., and Weyrich, A. S. (2001) J. Biol. Chem. 276, 529–543
29. Guthmann, F., Mayer, B., Koesling, D., Kukovetz, W. R., and Bohme, E. (1992) Biochim. Biophys. Acta 1100, 3–10
30. Bellamy, T. C., Griffiths, C., and Garralda, J. (2002) J. Biol. Chem. 277, 21666–2170
31. Mullershausen, F., Rusuwurm, M., Koelsch, D., and Friese, A. (2003) Vascul. Pharmacol. 40, 161–165
32. Kotera, J., Grimes, K. A., Corbin, J. D., and Francis, S. H. (2003) Biochem. J. 372, 419–426
33. Julius, D. M., Soderling, S., Burns, F., and Beavo, J. A. (1999) Rev. Physiol. Biochem. Pharmacol. 135, 67–104
34. Wood, K. S., and Ignarro, L. J. (1987) J. Biol. Chem. 262, 5020–5027
35. Zhou, H. L., and Torphy, T. J. (1991) J. Pharmacol. Exp. Ther. 258, 972–978
36. Keely, S. L., Jr., and Lincoln, T. M. (1978) Biochim. Biophys. Acta 543, 251–257
37. Stoehr, S. L., and Garthwaite, J. (2002) Am. J. Physiol. 282, 13049–13054