Nitric oxide activation of guanylyl cyclase in cells revisited

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Nitric oxide (NO) elicits physiological effects in cells largely by activating guanylyl cyclase (GC)-coupled receptors, leading to cGMP accumulation. Like other receptor-coupled effector mechanisms, NO stimulation of GC activity was previously considered to be a graded, concentration-dependent response, with deactivation following swiftly once the agonist disappeared. Recently, a new and unconventional mechanism has been proposed from experiments on purified protein [Cary, S. P. L., Winger, J. A. & Marletta, M. A. (2005) Proc. Natl. Acad. Sci. USA 102, 13064–13069]. It was concluded that GC in vivo will display a dual regulation by NO: a long-lasting tonic activity (10–20% of maximum) due to persistent occupancy by NO of the heme binding site and phasic activity due to engagement of another unidentified, lower affinity site. The hypothesis was first tested by monitoring GC activity in rat platelets maintained in vitro and exposed to calibrated NO transients. The kinetics was as expected for a single binding site for NO (EC50 = 10 nM), with activation and deactivation of enzyme activity conforming to the predictions of a simple receptor model. No tonic GC activity attributable to long-term NO binding was detected after exposure to the full range of active NO concentrations (peaking at 2–500 nM). Comparable results were obtained by using neural cells isolated from the cerebellum. After exposure to high NO concentrations, persistent GC activity could be recorded, but this activity was caused artifically by secondary NO sources being formed in the medium. The new scheme for regulation of GC activity by NO is of doubtful relevance to cells.

Results

Response of Platelets to NO Transients. The first experiments were carried out on rat platelets, which were used previously to analyze quantitatively NO-cGMP signaling and downstream activity of cGMP-dependent protein kinase under steady-state conditions (14). Being homogenous, of very small size, and rich in the αβ1 GC-coupled NO receptor isoform, these cells are ideal for addressing the present issue. The initial goal was to characterize the platelet cGMP response to calibrated NO transients, for which a new method was developed (Figs. 1 and 2; see also Materials and Methods). According to the hypothesis being examined (9), low NO concentrations will maximally generate 10–20% of the attainable GC activity, and this activation will persist because NO remains attached to the heme binding site on the receptor protein (half-life = 37 min at 10°C). Higher NO concentrations will engage the non-heme binding site and elicit dynamic regulation of the enzyme activity. For comparison, the extent to which the cellular data obtained here conform to predictions of the conventional scheme was analyzed by using a simple two-step receptor model having a single NO binding site (Fig. 1B; see also Materials and Methods).

Platelets were exposed to NO pulses lasting 10–20 s and varying in peak amplitude (1.7–500 nM) by adding a rapid NO releaser in the presence of a slow NO scavenger (Fig. 2). The NO donor was the proline/NO adduct (PROLI/NO, 5–1,500 nM), and the scavenger was 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO, 50 μM). At the low NO concentrations, cGMP was highest at the earliest time point examined (2 s, the peak in the presence of physiological concentrations of ATP and GTP, resulting in a tonic level of GC activity amounting to 10–20% of the maximum. Rapidly reversible GC activity of the type that has been found in cells (5) and which is likely to underlie the transient functional responses of cells to NO (10, 11) is suggested to be due to NO binding to another unidentified site of lower affinity but coupled to full enzyme activity. Thus, the receptor is viewed as providing both an enduring background synthesis of cGMP as well as a phasic response to momentary NO signals.

If correct, the new hypothesis would demand a reassessment of the way that NO functions (12). Before accepting the hypothesis, however, it is critical to test its applicability to the way that the receptor behaves in a cellular environment in which factors such as the local concentrations of ATP and GTP are under physiologic control and the protein is in its native state, possibly in a complex with other proteins (13). The experiments reported here were designed to test the hypothesis that cellular GC-coupled NO receptors exhibit the proposed dual regulation by NO.

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Abbreviations: PROLI/NO, proline/NO adduct; CPTIO, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; PDE, phosphodiesterase; GC, guanylyl cyclase.

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of the NO pulse) and then decayed to baseline within 10 s (Fig. 3A). With intermediate concentrations, the cGMP peak shifted to 5 s, whereas at high concentrations, it shifted back to 2 s. At all of the middle-to-high concentrations, cGMP fell abruptly after 5 s to attain a sustained low amplitude plateau after 10 s. The residual plateau is presumed to be a mixture of extracellular cGMP (14) and cGMP bound to proteins, notably cGMP-dependent protein kinase (15), both of which render cGMP inaccessible to the PDE.

The platelet cGMP response to NO is governed not only by activity of the receptor-associated GC activity but also by the rate of receptor desensitization and the activity of cGMP-degrading PDEs. The time courses of the changes of platelet cGMP with time and NO concentration could be accurately simulated by the two-step receptor model combined with desensitization and enhancement of PDE5 activity at rates determined previously from steady-state measurements (Fig. 3C).

The underlying changes in GC and PDE activities after delivery of a sample pulse of NO (50 nM PROLI/NO; peak NO = 17 nM) are illustrated in Fig. 3D. Except for the highest NO concentration (500 nM peak), plotting the amplitude of cGMP at the earliest time point (2 s) against the peak NO concentration gave a curve that matched the NO concentration-response curve observed under steady-state conditions in rat platelets (14), the EC50 being 11 nM and the Hill coefficient being 1.2 (Fig. 3B).

The experimental values are almost exactly as predicted by the receptor model (at the 2-s time point; EC50 for NO = 13 nM, Hill coefficient = 1.2). A larger Hill coefficient than expected for a single binding site (1.0) reflects distortion of the curve by GC desensitization and PDE enhancement. The reduced response at very high NO concentration (500 nM) is predominantly caused by desensitization (5), which, on average (Fig. 3B), was greater than in the experiment shown in Fig. 3A.

**Tests for Persistent NO-Stimulated GC Activity in Platelets.** These data, along with previous results obtained under steady-state conditions (14), provide no evidence for two NO binding sites differing in affinity. They do not exclude the possibility, however, that the affinities are too similar, or that the high affinity component is too small, to be resolved. Accordingly, we tested a second prediction of the two-binding site hypothesis (9), namely that transient exposure to lower NO concentrations should produce persistent GC activity amounting to 10–20% of the maximum. To test this prediction, platelets were exposed to NO transients, and then, after a suitable delay, the ongoing GC activity was measured by addition of the PDE5 inhibitor, sildenafil. When testing using low and high NO concentrations (peaks of 5 and 500 nM), the initial transient cGMP responses were as before (Fig. 4A). In the presence of sildenafil and the higher NO pulse, cGMP continued to accumulate for the duration of the pulse (10–20 s). This control response was similar when retested 60 s after a first exposure made in the absence of sildenafil, although the initial rate was greatly reduced (by 75%), signifying persisting receptor desensitization (14, 16). Addition of sildenafil 60 s after the initial lower amplitude NO transient (5 nM peak) generated no significant increase in cGMP, implying negligible residual GC activity. In contrast, with a high initial exposure to NO (500 nM peak), sildenafil addition brought about a clear increase in cGMP, the initial rate being ~10% of the maximum attainable at that time. This secondary activity was only seen after NO pulses that were saturating, or near saturating, for initial cGMP accumulation (Fig. 4B). The lowest NO concentration producing significant secondary activity above basal was one peaking at 50 nM, and the activity here was 1.5% of the maximum available, demonstrating the sensitivity of the detection method.

Basal GC activity was 0.17 pmol/mg of protein per s, a value that corresponds to ~0.1% of the apparent maximum rate measured after 2 s of stimulation with NO, or 0.07% of the predicted true maximal GC activity (see Fig. 4 legend).

The appearance of tonic GC activity after high amplitude pulses of NO could be consistent with the dual binding site hypothesis if it were assumed that persistent NO binding required a high priming NO concentration. However, the secondary activity was not caused by NO remaining bound to its receptors but by persisting free NO released from secondary donors formed in the incubation medium. An important ingredient for generation of the secondary NO sources was Heps buffer (see Fig. 6, which is published as supporting information on the PNAS web site).

These results indicate that preexposure of platelets to the whole range of active NO concentrations fails to induce any persistent GC activity attributable to long-term NO binding. In case this negative finding may have been influenced by the manner of NO delivery, an alternative method was used. In this method, PROLI/NO (350 nM) was added to the platelet suspension (without CPTIO or urate) to give a supramaximal NO concentration (500 nM) and, after 20 s, hemoglobin (100 μM) was added to remove free NO. Subsequent addition of sildenafil revealed no detectable GC activity (see Fig. 7, which is published as supporting information on the PNAS web site).

**Experiments in Cerebellar Cells.** Finally, in case platelets are abnormal, we tested another cell type. Astrocytes from the cere-
bellum generate high amplitude, long-lasting cGMP responses to NO because they have an abundance of NO-stimulated GC activity but very little cGMP-degrading PDE activity (17). In suspensions of cerebellar cells, in which astrocytes are the only relevant cell type (5), the NO concentration-cGMP response curve to NO given in the form of transients in a Tris-buffered medium (Fig. 5A) was similar to that in platelets (EC50 ≈ 10 nM NO) but, as reported before (5), the curve was steep and biphasic because of desensitization. With a maximally effective NO pulse (100 nM peak), cGMP rose to level off after ≈20 s (the end of the pulse) and then fell very gradually, reflecting the low PDE activity (Fig. 5B). Three minutes after the first pulse, when cGMP was still elevated, a second NO pulse given in the presence of appropriate PDE inhibitors (rolipram plus sildenafil) gave a clear second response although, as usual, the initial rate and amplitude were reduced because of persistent desensitization (50% loss of GC activity at this time point). Addition of the PDE inhibitors alone, however, gave no measurable rise in cGMP but simply stopped the levels from falling, showing that there was no significant tonic GC activity (a residual 10% of the available GC activity would have been easily detected; Fig. 5B, dashed line).

**Discussion**

The dual NO binding site hypothesis for stimulation of GC activity (9), formulated from experiments carried out with or without ATP and GTP in a cell-free environment, predicted a fundamentally different mechanism of regulation of cellular function by NO in vivo compared with the currently accepted scheme (2, 4, 18). In two different cell types, however, we found no evidence for two distinct binding sites on the basis of

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**Fig. 3.** cGMP responses to NO pulses in rat platelets. (A) Time courses of cGMP formation by rat platelets in response to a range of NO transients (peak concentration indicated on each set of data). (B) Concentration dependence measured by exposing platelets to NO transients for 2 s. The solid line fits the data (excluding the highest NO concentration) to the Hill equation; nH represents the Hill coefficient. (C) Analysis of the data in B by the receptor model (Fig. 18). Parameter values were the following: GCmax = 240 pmol/mg of protein per s; Vp(tot) = 257 pmol/mg of protein per s; for 1.7, 5, and 17 nM peak NO, k0 = 0.03, 0.05, and 0.11 s⁻¹, respectively, and kd = 0; and for 50, 150, and 500 nM peak NO, k0 = 0.15 s⁻¹ and kd = 0.05, 0.13, and 0.23 s⁻¹, respectively. For the sake of completeness, extracellular/bound cGMP (represented by the plateau seen after 10 s) was assumed to be formed exponentially with a rate constant of 0.5 s⁻¹ (14). (D) Kinetics of NO (dashed line), active NO-bound GC (labeled GC), PDE, and cGMP (thicker line) predicted by the model for a sample NO pulse. The units for GC and PDE activities are picomoles per milligram of protein per second and, for cGMP, picomoles per milligram of protein.

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**Fig. 4.** Test for tonic GC activity after stimulation of rat platelets with NO. (A) Pulses of NO (5 and 500 nM peak) were delivered to the platelets at t = 0, and sildenafil (sild, 100 μM) was added after 60 s to reveal any continuing cGMP formation relative to the maximum rate achieved by delivery of a further NO pulse (500 nM peak) in the presence of sildenafil at the same time point (Control 2). The equivalent response at the start was also monitored (Control 1). This response and the initial responses to the NO pulses were fitted by the receptor model (Fig. 18) using the following parameters: GCmax = 235 pmol/mg of protein per s; Vp(tot) = 257 pmol/mg of protein per s; k0 = 0.035 and 0.15 s⁻¹ (5 and 500 nM NO, respectively); and kd = 0 and 0.23 s⁻¹ (5 and 500 nM NO, respectively). To fit the second control data (Control 2), GCmax was reduced to 60 pmol/mg of protein per s, and it was assumed that there was no further desensitization (i.e., k0 = 0). (B) NO concentration-response curves for the primary and secondary GC activity measured with 2 s exposures (1° response) or from the slope of the initial rise in cGMP after addition of sildenafil (2° response). The latter was based on data gathered every 20 s for 120 s. **, P < 0.01 and ***, P < 0.001 compared with basal activity.
concentration-response relationships, in line with previous findings under steady-state conditions in intact platelets (14), in lysates of platelets and cerebellar cells (19), in lysates of cells expressing either the α1β1 or α2β1 GC isofoms (20), or with NO-activated GC purified from lung (20). More tellingly, when free NO was removed, no tonic activity (above basal) was detectable after exposures to NO covering the whole spectrum of active concentrations. This inactivity was not because basal GC was already in a tonically activated state: in the platelets, basal activity was 0.05–0.1% of maximum, which is much lower than typically found in purified enzyme preparations (0.5%). The experiments cannot rule out there being a second NO binding site. They do, nevertheless, cast doubt on the idea that GC activity in cells is under the type of dual regulation by NO (tonic and phasic) for which the existence of two distinct NO binding sites was hypothesized.

Unsurprisingly, a number of differences between the behavior of NO-stimulated GC activity in cell-free systems and in intact cells have already become apparent (reviewed in ref. 8). In cell-free conditions, NO has a 10-fold higher potency and, probably related to this, the enzyme deactivates 10-fold more slowly upon removal of NO. In cells, GC activity undergoes rapid desensitization, a property that disappears upon lysis. Desensitization had previously only been studied during or after prolonged exposure of cells to NO, but, for the time being, it appears to provide a robust starting point. The question of the tonic and phasic actions of NO found in different tissues, for which the new scheme for GC function was deemed to provide an explanation (12), may be better approached from the perspective of NO synthases, which are known to be capable of generating NO tonically and phasically (27).

Materials and Methods

Materials. CPTIO and PROLI/NO were obtained from Axorra (UK) Ltd. (Nottingham, U.K.), and sildenafil was supplied by the Chemistry Division of the Wolfson Institute for Biomedical Research (London, U.K.). Other special chemicals were from Sigma-Aldrich (Poole, Dorset, U.K.).

Cell Preparation. Rat platelets and suspensions of cells from the rat cerebellum were prepared and incubated as described in refs. 14 and 16. The platelet medium comprised the following (in mM): NaCl (137), MgCl2 (0.5), NaH2PO4 (0.55), KCl (2.7), Heps (25), and D-glucose (5.6), pH 7.4 at 37°C, and the cell concentration was 0.5 mg of protein per ml. In some experiments (Fig. 6), Tris (25 mM) or Na2HPO4 (25 mM) was substituted for...
Hepes, with the remainder of the solution staying the same. Cerebellar cells were incubated at 20 million cells per ml in a solution containing the following (in mM): NaCl (130), KCl (3), MgSO\(_4\) (1.2), Na\(_2\)HPO\(_4\) (1.2), Tris (15), CaCl\(_2\) (1.5), and glucose (11), pH 7.4 at 37°C. With both platelets and cerebellar cells, L-nitroarginine (100 \(\mu\)M) was included to remove possible complications arising from endogenous NO formation.

**Measurement of NO.** NO concentrations were recorded at 1 Hz sampling frequency at 37°C in a sealed, stirred vessel equipped with an electrochemical probe (ISO-NO Mk II, World Precision Instruments, Sarasota, FL).

**Delivery of NO Transients.** To test for persistent NO-stimulated GC activity, a method for exposing the cells to NO transients of known amplitude and duration was developed. The method extends the one used previously to administer constant, of known amplitude and duration was developed. The method extends the one used previously to administer constant, clamp NO concentrations (20) and is based on balancing a source that releases NO with a sink that consumes it (Fig. 1A). In this case, the NO source was the NONOate, PROLI/NO, which decomposes rapidly (half-life = 1.8 s at 37°C and pH 7.4) and the sink was (as before) CPTIO, which reacts with NO at a suitable rate. The product of the reaction, the NO\(_2\) radical, was converted to nitrite by including urate at a physiological concentration (300 \(\mu\)M). To give NO exposures lasting 10–20 s, a CPTIO concentration of 50 \(\mu\)M was selected. Modeling (Fig. 2A) predicts that the peak of NO occurs at \(\approx 2\) s and is proportional to PROLI/NO concentration, after which the concentration declines effectively to zero by 20 s. Without a sink, a prolonged NO plateau is generated. Measurements of the NO concentration by using an electrochemical probe accorded closely with expectations (Fig. 2B) except that, in the presence of CPTIO, the times of the peak and return to baseline were delayed by a few seconds. The slowing is due to the electrode response time (28). The measured peak NO concentrations and their linear dependence on PROLI/NO concentration were, however, essentially identical to predictions, assuming a yield of 1.5 molecules of NO per PROLI/NO molecule (Fig. 2B, Inset). Thus, the method fulfills requirements for delivery of calibrated NO transients. In each experiment, the peak NO concentration achieved by 1,500 nM PROLI/NO was measured. It averaged 485 \(\pm\) 13 nM (\(n = 9\)) and so, given the proportional relationship (Fig. 2B, Inset), the peak NO concentration was taken to be a third of the donor concentration (to be exact, the predicted proportionality constant is 0.37). Importantly, the method allows, for the first time, precise NO transients to be delivered repeatedly to the same preparation, because each application of PROLI/NO only uses up a small fraction of the available CPTIO. For example, after 10 successive applications of 150 nM PROLI/NO, the predicted peak NO concentration is only 1 nM higher than at the start (55 nM), a prediction that agrees with experimental observations (data not shown).

For use with cells, unless stated otherwise, CPTIO and urate were added to the cell suspensions together with superoxide dismutase (1,000 units/ml) to remove any superoxide ions that would otherwise react with NO. PROLI/NO was then added with rapid mixing, and 50-\(\mu\)l aliquots of the suspension were withdrawn at various intervals and inactivated by addition to 200 \(\mu\)l of boiling buffer (50 mM Tris and 4 mM EDTA, pH 7.4). At least three independent runs of each condition were carried out in each experiment. Levels of cGMP were measured by RIA.

When used, hemoglobin was added in its reduced, oxygen-bound form from concentrated stock solutions (29).

**Modeling Cellular cGMP Responses to NO.** The scheme in Fig. 1A was used to generate NO profiles resulting from the PROLI/NO-CPTIO mixture as described in ref. 20. For modeling the subsequent cellular cGMP response, the scheme used (Fig. 1B) was the one developed beforehand (14), with the addition of explicit rate constants for the steps in receptor activation. The values of the rate constants were selected to accommodate all of the known properties of NO-activated GC activity in cells, including NO concentration-dependence, the rates of activation and deactivation, and the efficacy of NO (8). With this modification, the model permits simulations to be performed under the nonequilibrium conditions used in the present experiments. In the model, the level of cGMP is the difference between the rate of synthesis, which is assumed to be proportional to the amount of receptor in its NO-bound activated form (NO\(_{GC}^+\) in Fig. 1B), and the rate of hydrolysis by PDEs. The rates of cGMP synthesis and hydrolysis are modified over time by receptor desensitization and, in the case of platelets, by enhancement of the activity of the operative PDE (PDE5).

The rate constants for desensitization (k\(d\)) and for enhancement of PDE5 activity (k\(e\)) were obtained from steady-state measurements in rat platelets (14). Inhibition of PDE5 by sildenaif in platelets was incorporated into the model by using the experimentally determined value of the inhibitory constant; the limiting GC activity was adjusted according to the amplitude of the maximal cGMP response in any particular experiment and covered the range of values determined previously; the total limiting PDE5 activity in platelets was assumed to comprise two components, one present under basal conditions and one that became enhanced with time, the limiting activity of the former being fixed at one-sixth of the latter (14).

For cerebellar cells, and conforming to experimental observations (17), the PDE activity was assumed to be a mixture of PDE5 (having the same kinetic properties as the platelet PDE5) and PDE4, which was taken to have an affinity constant for cGMP of 1 mM and a time-independent activity. The inhibitory constant for rolipram on PDE4 was taken to be 30 nM, and the relative proportions of PDE5 and PDE4 were adjusted to simulate the observed decay of cGMP in the absence of NO. For calculations, the responsive cells (the population of astrocytes) were assumed to be 5 \(\mu\)m in radius and to represent 6% of the total cell number (17), so 1 pmol cGMP/10\(^6\) cells converts to 32 \(\mu\)M cGMP in the astrocytes.

Parameter values used in all simulations are given in the appropriate figure legends, wherein the limiting GC activity is abbreviated as GC\(_{max}\) and the total limiting PDE activity as \(I_p(tot)\). Equations were solved numerically by using the adaptive Runge–Kutta algorithm in Mathcad 11 (Adept Scientific, Letchworth, Herts, U.K.). A sample worksheet is available from J.G. upon request.

**Statistics.** Data are presented as means \(\pm\) SEM and, where appropriate, were analyzed for significance by using Dunn’s Multiple Comparisons test in Instat (GraphPad, San Diego, CA); \(P < 0.05\) was considered significant.

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