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Sub-second Kinetics of the Nitric Oxide Receptor, Soluble Guanylyl Cyclase, in Intact Cerebellar Cells*

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Tomas C. Bellamy and John Garthwaite‡

From the Wolfson Institute for Biomedical Research, University College London, Cruciform Building, Gower Street, London WC1E 6BT, United Kingdom

Soluble guanylyl cyclase (sGC) catalyzes cGMP synthesis and serves as a physiological receptor for nitric oxide (NO). Recent evidence indicates that key properties of sGC within cells differ from those of purified sGC. We have devised a technique for resolving NO-stimulated sGC activity in cells on a sub-second time scale, enabling the first quantitative description of the kinetics of the enzyme within its natural environment. Upon release of NO from a caged derivative, sGC became activated without any lag observable at a 20-ms sampling time. Deactivation of sGC on removal of NO occurred with a rate constant of 3.7 s^{-1}, which is 25-fold faster than the fastest estimate for purified sGC. Desensitization of sGC occurred with a time constant of 6.9 s at an estimated 70 nM NO and became faster at a higher concentration, indicating that NO accelerates desensitization. The concentration-response curve for NO consequently became increasingly bell-shaped with time, a phenomenon that causes the apparent potency of NO to increase with time. The results indicate that sGC within cells behaves in a highly dynamic fashion, allowing the NO-cGMP pathway to operate within a kinetic framework more resembling that of neurotransmission than the properties of purified sGC suggest.

Nitric oxide (NO)[1] performs diverse biological functions, ranging from relaxation of smooth muscle and inhibition of platelet aggregation to neural signaling in the peripheral and central nervous systems (1, 2). The principal receptor mediating the physiological actions of NO is the cGMP-synthesizing enzyme, soluble guanylyl cyclase (sGC). Exposure to NO increases the sGC catalytic activity up to several hundred-fold (3–5), and the resulting cellular accumulation of cGMP can engage a number of downstream targets, including cGMP-dependent protein kinase (6), cGMP-regulated phosphodiesterases (7), and cyclic nucleotide-gated ion channels (8) to bring about the various biological effects.

Since the purification of sGC in the late 1970s, much knowledge of the structure and the mechanism of activation of the enzyme by NO has accrued (9). The enzyme is an $\alpha\beta$-heterodimer with a prosthetic heme group, the NO binding site, attached to the β-subunit. Somewhat unexpectedly by comparison with analogous receptors, sGC exhibits limited molecular heterogeneity; only 2 α- and 2 β-subunits have been identified, and only 2 isoforms have so far been found to exist at the protein level as follows: $\alpha_1\beta_1$, which is widespread, and $\alpha_2\beta_1$, which is found in human placenta (10). The two isoforms appear to have similar functional and pharmacological properties (10).

As with any other signaling molecule, knowledge of the kinetic properties of the receptor is a prerequisite for understanding how the signals are decoded. When studied in tissue homogenates or in its purified form, sGC exhibits simple Michaelis-Menten-type kinetics. Consequently, in the presence of NO, excess substrate (GTP), and cofactor (Mg^{2+}), sGC synthesizes cGMP at a constant rate over long periods, more in the manner of a "housekeeping" enzyme than a receptor. However, our recent investigation (11) of how sGC behaves within its natural cellular environment suggested a very different profile of activity; within seconds of adding NO, the enzyme underwent rapid desensitization to reach a steady-state level of activity that was 10–15% of the peak. Other kinetic properties of sGC were also different in the cells compared with those of the purified enzyme, notably the potency of NO and the rate at which the enzyme deactivated following removal of NO.

The precision of this previous study was handicapped by the poor temporal resolution of the method used (simple manual pipetting), and so much of the information had to be extracted from the data by mathematical deconvolution techniques. Furthermore, the values of key kinetic parameters were not disclosed because they were too fast to be measured. To address these deficiencies, we have devised a method for investigating second messenger responses in cells on a sub-second time scale. The aim was to determine the kinetic constants for sGC activation, deactivation, and desensitization, and so obtain the first quantitative description of NO-stimulated sGC activity in a physiologically relevant setting.

EXPERIMENTAL PROCEDURES

Cerebellar cell suspensions were prepared from 8- to 9-day-old rats as described previously (11) and were incubated at a concentration of 20×10^6 cells/ml at 37 °C. From at least 1 h before use, the NO synthase inhibitor L-nitroarginine (100 μM) was included to prevent possible complications arising from endogenous NO production. Aliquots (200 μl) of the cells were added to a glass vessel (V005; QM_x Laboratories, Essex, UK) and preincubated for 5–10 min with the phosphodiesterase inhibitor sildenafil citrate (100 μM). This concentration is sufficient to inhibit completely phosphodiesterase activity in these cells at the cGMP levels achieved in the present experiments (12). The vessel was placed in the rapid-quenching apparatus (Fig. 1) after addition of the UV-sensitive compound potassium ruthenium nitrosyl pentachloride (13), hereafter referred to as "caged-NO," to the suspension.

Rapid Quenching of Cells—The apparatus (Fig. 1*a*) consists of a perspex module, with a central port surrounded by a water jacket at

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‡ To whom correspondence should be addressed. Tel.: 00 44 20 7679 6694; Fax: 00 44 20 7209 0470; Email: john.garthwaite@ucl.ac.uk.

[1] The abbreviations used are: NO, nitric oxide; sGC, soluble guanylyl cyclase; caged-NO, potassium ruthenium nitrosyl pentachloride; caged-ATP, ATP, $P^3$1,-(2-nitrophenyl)ethyl ester (disodium salt); GFAP, glial fibrillary acidic protein; DAPI, 4′,6-diamidino-2-phenylindole; P.D., potential difference.

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37 °C. The glass vessel sits within the port, over a photodiode (OSD15-E visible light enhanced; RS Components, Northants, UK). Above the vessel, a XF-10 xenon flash lamp (Hi-Tech Scientific; Salisbury, UK) fitted with a UG11 filter (peak transmission 320 nm) was mounted at a distance of ~15 cm from the bottom of the vessel. The focal point for the lamp was only 2.5 cm so that, on flashing, the cell suspension was exposed to an unfocused pulse of UV light. Just above the vessel, either one or two glass pipettes (tip internal diameter ~ 350–400 μm), formed with an electrode puller, were positioned obliquely with micromanipulators. These pipettes were attached to a Picospritzer (Picospritzer IID, General Valve Corp.).

The principle of the machine is simple; at a predetermined time after uncaging NO with an UV flash (intensity, 200 V, duration 1 ms), a volume (20 μl) of 3 M trichloroacetic acid is expelled from the pipette under pressure (50 pounds/square inch, 10-ms pulse duration) and mixes with the cell suspension (final trichloroacetic acid concentration = 0.3 M), denaturing the cells and therefore quenching the cGMP accumulation. The photolysis time of the caged-NO is <1 ms (13) and estimated quenching time is <3 ms (14). The triggering of the flash lamp and Picospritzer is coordinated by a Master 8 voltage step generator (Intracel Ltd., Hertfordshire, UK). The vessel was then removed from the apparatus, and the sample was neutralized with an equal volume of 1,1,2-trichlorofluoroethane:tri-*n*-octylamine (4:1 v/v), mixed thoroughly and spun at 2500 × *g* for 10 min. The upper (aqueous) layer was removed and its cGMP content measured by radioimmunoassay.

To evaluate the method in terms of speed and thoroughness of mixing, 20 μl of India ink was injected into 200 μl of incubation buffer. The resulting change in light intensity was registered as a change in potential difference (P.D.) across the photodiode (response time 12 ns). The P.D. was monitored with an oscilloscope and captured on computer. The mean change in P.D. with time is illustrated in Fig. 1*b*. There was a 15-ms delay from triggering of the Picospritzer to onset of mixing (presumably attributable to the time taken for the propagation of the pressure pulse). The subsequent total mixing time was ~5 ms. In the data presented, all time points take the 15-ms lag into consideration (*i.e.* they represent the time of onset of mixing).

In experiments aimed at determining the rate of sGC deactivation, a second pipette was used to inject either incubation buffer (for control cells) or hemoglobin (final concentration 100 μM) to scavenge NO. With this excess of Hb over NO, free NO would disappear with a half-time of less than 50 μs (15). The cells were subsequently quenched with trichloroacetic acid at fixed intervals.

The data are presented as means ± S.E. for 3–4 independent samples at each time point in each experiment.

For identifying cGMP accumulating cells by immunocytochemistry, paraformaldehyde was injected in place of trichloroacetic acid, to give a final concentration of 4%. The samples were allowed to fix for 15 min, and they were then processed as described previously (11), except that the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) was present in the mounting medium to illustrate the total cell number in a given field. Slides were viewed with a confocal microscope (TCS SP; Leica, Heidelberg, Germany).

Estimation of NO Released from Caged-NO—To estimate the concentration of NO achieved following flash photolysis, the method of Murphy *et al.* (13) was adapted. Caged-ATP (ATP, $P^3$1,-(2-nitrophenyl)ethyl ester, disodium salt) was added to 200 μl of freeze-fractured cells, which had been boiled for 20 min, sonicated, and vortex-mixed. Following flash photolysis, the samples were kept on ice, and the ATP was assayed (in subdued light) by the firefly luciferase method ("ATP-lite-M" kit, Packard Instrument Co.). Un-flashed samples were assayed as a control, and the values were deducted from the total ATP in the samples, to give the concentration of uncaged ATP. As the caged-ATP and caged-NO compounds have nearly identical absorption spectra in the range of filtered flash lamp wavelengths, the amount of uncaged NO can be deduced from the relative quantum efficiencies of the cages, which is 0.08 (13). With the above protocol, 2.96% of ATP was uncaged per flash, indicating that 0.237% of NO would be uncaged. This value was used for estimating the NO concentrations achieved in the experiments.

Materials—Potassium ruthenium nitrosyl pentachloride and ATP, $P^3$1,-(2-nitrophenyl)ethyl ester (disodium salt) were from Molecular Probes (Leiden, The Netherlands); L-nitroarginine was from Tocris Cookson (Bristol, UK); sildenafil citrate was supplied by the Chemistry Division, Wolfson Institute for Biomedical Research. All other special chemicals were from Sigma.

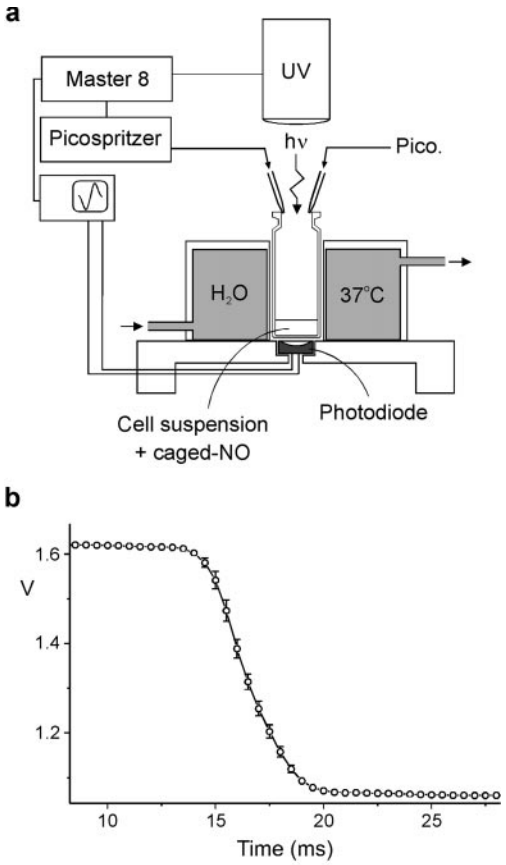

FIG. 1. **Apparatus for rapid quenching of cells.** *a*, a glass vessel containing 200 μl of the cell suspension (+ caged-NO) is situated within the port of a perspex module, surrounded by a 37 °C water jacket. A UV pulse uncages a fixed amount of NO, initiating sGC activation. After fixed time intervals, 20 μl of 3 M trichloroacetic acid is expelled under pressure from a pipette attached to a Picospritzer (*Pico*). Timing is coordinated by a Master 8 voltage-step generator. To determine mixing time, India ink (20 μl) is ejected from the pipette into a vessel containing buffer (200 μl), at time = 0. This results in a change in potential difference across a photodiode mounted beneath the vessel, which is measured with an oscilloscope. The mean of 6 such runs (± S.E.) is illustrated in *b*.

RESULTS

The purpose-built rapid quenching apparatus (Fig. 1 and "Experimental Procedures") allowed the termination of cGMP accumulations in the cell suspensions with a mixing time of ~5 ms and quenching time <3 ms. In applying the technique to the measurement of sGC kinetics, therefore, an operational limit of 20-ms intervals between data points was imposed.

Because the experimental material was a cell suspension containing different cell types, it was important in the first instance to identify the cells generating cGMP in response to NO over the time scales used for subsequent biochemical measurements. Previous immunocytochemical studies with the cell suspension had shown that the cGMP accumulations were restricted to a subpopulation of cells, the astrocytes, regardless of whether the cells were exposed to NO for 10 s or 2 min (11). However, given that certain cells (*e.g.* human platelets) exhibit a transient cGMP signal (11, 16), it was important to ascertain whether any other cell types in our preparation were making a significant contribution with sub-second exposures to NO. To address this, paraformaldehyde was injected into the cell suspension (final concentration, 4%) to fix the cells 100 ms to 1 s after uncaging NO (from 30 μM caged-NO). The samples were then double-stained for the astrocytic marker, glial fibrillary acidic protein (GFAP), and for cGMP. In control cells, only the occasional cell stained for cGMP, and such cells were also

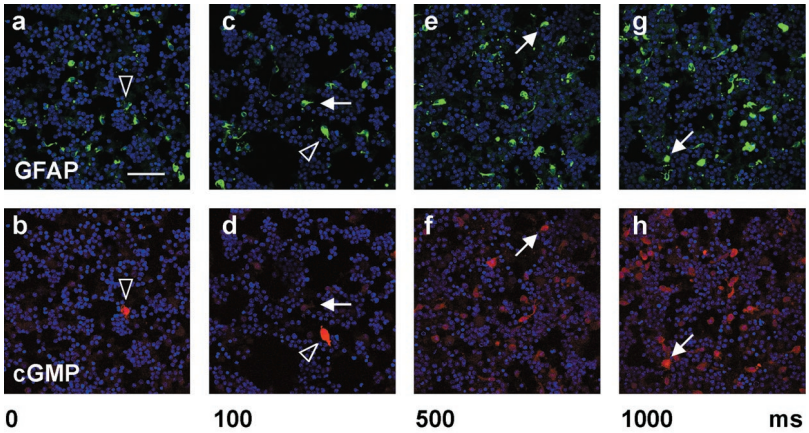

FIG. 2. **Location of cGMP accumulations in cell suspensions over 1 s.** Cells were stained for GFAP (*green, a, c, e,* and *g*) and cGMP (*red, b, d, f,* and *h*), and counterstained with the nuclear stain DAPI (*blue*). Cells were fixed at 0 (*a* and *b*), 100 (*c* and *d*), 500 (*e* and *f*), and 1000 (*g* and *h*) ms after uncaging of NO. *Arrows* indicate examples of colocalized staining. Occasional cells contained high levels of cGMP even under unstimulated conditions (*arrowheads*). *Bar* = 50 μm.

GFAP-positive (Fig. 2, *a* and *b*). After 100 ms of exposure to NO, additional cGMP-positive cells were present, although the staining was relatively weak. Again, cGMP immunoreactivity could not be identified in cells that were not GFAP-positive (Fig. 2, *c* and *d*). The same result applied when the NO exposures were 500 ms (Fig. 2, *e* and *f*) or 1 s (Fig. 2, *g* and *h*), although the cGMP staining became increasingly intense. It is reasonable to assume, therefore, that no cell types other than astrocytes were contributing significantly to the measured cGMP elevations.

Rate of sGC Activation—The levels of cGMP were measured in cells quenched at different times following flash photolysis of a concentration (30 μM) of caged-NO that was maximally effective when assessed at 1- or 10-s time points (see below for concentration-response relationships). The most detailed time course (Fig. 3*a*) covered 0–400 ms, and this included measurements made every 20 ms for the first 200 ms. Over this period, the rate of cGMP accumulation appeared roughly linear; the first time point at which cGMP was significantly higher than the basal level was 40 ms ($p = 0.001$ by Student's *t* test). No lag in the onset of cGMP accumulation following liberation of NO could be discerned.

Kinetics of sGC Desensitization—Over the first 400 ms of sGC activity (Fig. 3*a*), there was no clear deviation from linearity in the rate of cGMP accumulation, but over a 1-s time course (Fig. 3*b*) the rate became noticeably nonlinear, an effect that became increasingly evident over a 10-s time course (Fig. 3*c*). Since the experiments were done under conditions where degradation of cGMP was abolished (see "Experimental Procedures") the progressive fall in the rate of cGMP accumulation reflects the progressive desensitization of sGC activity. To describe the cGMP accumulation with time, the data from the different time courses were combined into a single progress curve. This was rendered feasible without having to resort to a normalization procedure because of the small inter-experiment variation in the absolute values of the cGMP responses obtained using this technique. The combined data could be adequately fit by a single exponential function, having a time constant (τ) of 6.9 s for sGC desensitization; this fit is overlaid on all the data sets in Fig. 3, *a–c* (*solid line*). Whereas the single exponential provided a reasonable approximation of the time course as a whole, in no experiment did the line pass through the starting cGMP level (Fig. 3*a*). In fact, all 9 individual cGMP measurements at this time point fell below the line, indicating a significant misfit ($p < 0.05$ by sign test), which suggests that there may initially be an additional fast component to desensitization. The inclusion of a second early exponential (68 ms time constant) provides a fit to the starting data (not shown).

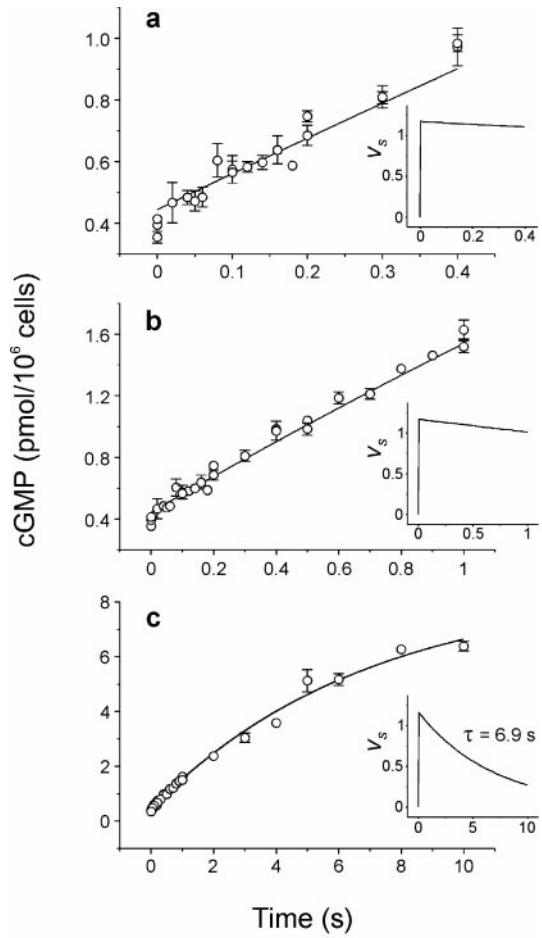

FIG. 3. **Kinetics of sGC activation and desensitization.** Cells were quenched over 3 time courses (*a–c*), following photolysis of 30 μM caged-NO at $t = 0$. *Solid lines* show an exponential function fitted to the combined data from the three experiments. *Insets* show the rate of cGMP synthesis (v_s) over the corresponding times (with $v_s = 0$, at $t = 0$). The time constant for desensitization (τ) was 6.9 s.

Rate of sGC Deactivation—Deactivation refers to the cessation of sGC activity after the agonist is removed. To investigate the rate of deactivation, NO was liberated (30 μM caged-NO) and cGMP accumulation allowed to proceed for 400 ms. A high

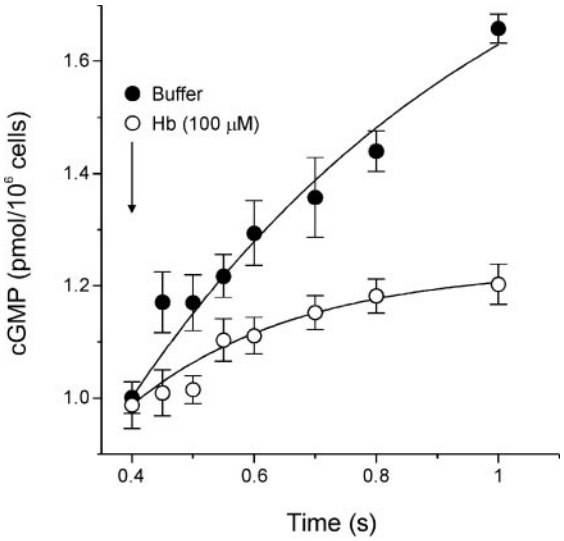

FIG. 4. **Kinetics of sGC deactivation.** 20 μl of buffer ( ) or Hb (○) were injected (*arrow*) 400 ms after photolysis of 30 μM caged-NO. Data are fit with exponentials (*solid lines*).

concentration of Hb (100 μM) was then added to scavenge the NO in the bathing medium (scavenging would be achieved in well under 1 ms). As a check, Hb was injected 20 ms before the UV pulse, and the usual cGMP accumulation was eliminated (data not shown). The inability of Hb to penetrate the cells means that the protein cannot directly influence sGC, and so the rate at which cGMP accumulation levels off (phosphodiesterase activity being inhibited) corresponds to the rate of deactivation of sGC. Control cells, which received an equivalent injection of buffer after 400 ms, continued to accumulate cGMP as normal (Fig. 4, *cf.* Fig. 3). On adding Hb, there was a rapid reduction in the rate of cGMP accumulation such that the first time point examined (50 ms later) was significantly less than control ($p < 0.05$ by Student's t test). By about 600 ms, cGMP was stable (Fig. 4). The overall time course could be fit by an exponential function corresponding to a deactivation rate constant of 3.7 s^{-1}. This must be regarded as a purely operational description, however, as the starting gradient of the fitted exponential (at 400 ms) was less than that of the control, implying that the true kinetics of deactivation is more complex and may involve an initial fast phase.

Concentration-Response Curves for NO Activation of Cellular sGC—Concentration-response relationships for NO activation of sGC were assessed using different concentrations of caged-NO and different periods of exposure to the liberated NO. At the first time point examined (100 ms after photolysis), cGMP increased in a simple sigmoidal manner with logarithmic increases in caged-NO concentration, the EC_{50} being about 20 μM and the maximum being reached at 100 μM (Fig. 5, *a* and *b*). The corresponding concentrations of released NO, estimated by comparing the quantum efficiency of the caged-NO and caged-ATP (see "Experimental Procedures"), are 47 and 240 nM. When examined after 1 s stimulation, and more so after 10 s stimulation, however, the concentration-response curves became bell-shaped. Moreover, at both time points, the maximum response occurred at the lower concentration of 30 μM (71 nM NO) and the EC_{50} was, accordingly, left-shifted to about 10 μM (24 nM NO). These changes in the concentration-response curves with time are what would be predicted for agonist-induced desensitization (17, 18). Such an effect is well illustrated by the progressive decline in the rate of cGMP accumulation over the interval 100 ms to 1 s relative to the initial rate (0–100 ms) as the NO concentration is raised (Fig. 5*c*). Accordingly, the potency of NO at the earliest time point (100 ms)

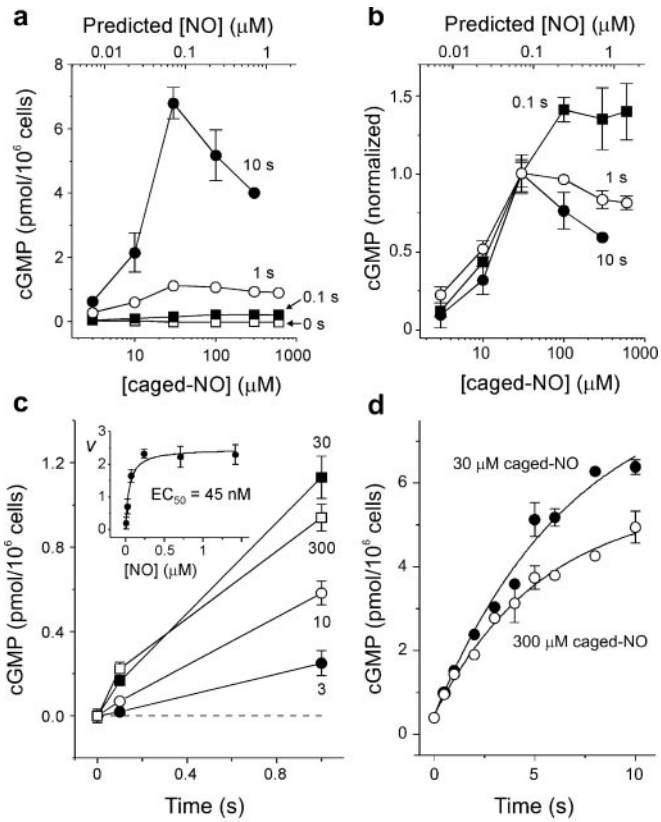

FIG. 5. **Concentration-response relationships for activation of sGC by NO.** *a*, cGMP levels at 0 (□), 0.1 (■), 1 (○), and 10 s ( ) after uncaging of NO. *b*, cGMP scaled to levels at 30 μM caged-NO at the same time intervals as in *a* after subtraction of background levels. *c*, cGMP levels (minus background) 0.1 and 1 s after photolysis of 3 ( ), 10 (○), 30 (■), and 300 μM (□) caged-NO; *inset*, rate of synthesis (v, pmol/10^6 cells/s) of cGMP by minimally desensitized sGC (100 ms after photolysis) against predicted NO concentration, fit by a standard Hill equation (with Hill constant = 1). *d*, time course of cGMP accumulations at 30 μM ( ) and 300 μM (○) caged-NO.

should correspond to that at which sGC desensitization exerts the least influence. The variation in this initial rate of cGMP formation with NO concentration (estimated) could be adequately fit by a standard Hill plot, which showed an EC_{50} for NO of 45 nM (Fig. 5*c*, *inset*).

To examine quantitatively how the rate of desensitization is influenced by agonist concentration, the time course of cGMP accumulation over 10 s at a high concentration of caged-NO (300 μM; 710 nM NO) was examined in more detail. As predicted, the rate of cGMP accumulation at the higher concentration was slower, indicating an enhanced rate of desensitization (Fig. 5*d*). An exponential fit to the data indicated a shortening of the time constant for desensitization from 6.9 to 5.4 s.

DISCUSSION

The development of a new method for rapid quenching of cell suspensions has enabled cGMP responses in cerebellar cells to be satisfactorily resolved on a sub-second time scale. The major factor limiting the time resolution of the method is the mixing time (5 ms). In principle, the approach can be applied to any cell type that can be kept in suspension, although it could be adapted easily for studying cells adhering to a solid surface, *e.g.* cells cultured on coverslips. Rapid changes in the levels of a variety of metabolites, second messengers, etc. could be measured. The apparatus is fairly inexpensive and relatively simple to construct and use, and it allows highly reproducible results from one day to another. Initially, attempts were made to use a

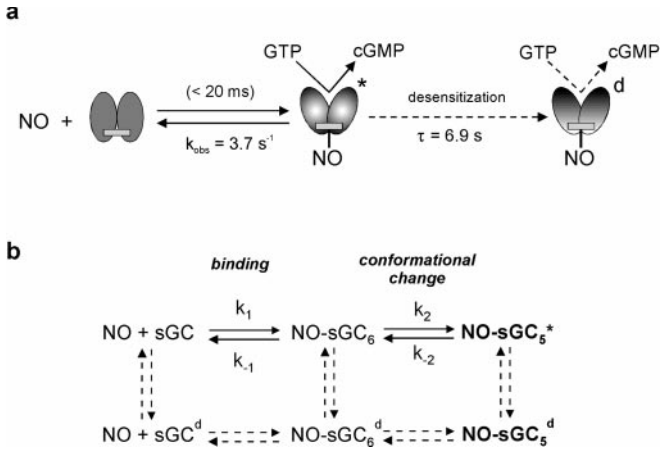

FIG. 6. **Models for sGC activity.** *a,* simplified scheme describing operational constants for sGC in intact cells. NO and sGC are in equilibrium between bound and unbound states. The rate of the forward reaction is too high to measure (<20 ms at 71 nM NO), but the constant (k_{obs}) for the back reaction (sGC deactivation) = 3.7 s^{-1}. Binding of NO forms the highly activated state (*), which becomes desensitized over time (τ = 6.9 s, k = 0.14 s^{-1}). *b,* scheme depicting the molecular details possibly underlying sGC kinetics. *NO-sGC$_6$* = 6-coordinate intermediate; *NO-sGC$_5$** = 5-coordinate active form; *sGC^d* = desensitized sGC. *Dashed lines* represent potential routes for transition to the desensitized form of the enzyme.

classical, commercially available, quench flow device, as used previously for measurements of cyclic nucleotides in olfactory cilia (19). However, in practice, the quench flow apparatus was found to have several disadvantages. For example, the time ranges permissible within a given experiment are limited, relatively large volumes of cell suspensions are needed, and the cells tend to settle out within the apparatus, causing variation in the numbers sampled. Furthermore, whereas small structures such as olfactory cilia may survive well, the forcing of whole cells under pressure through narrow-bore tubing creates potential problems associated with shear stresses (which vary in magnitude and duration with reaction time). With the present technique, the absolute values for cGMP in cells exposed to NO are closely similar to those reported previously from the use of simpler methods (11), suggesting the new method does not compromise the functional properties of the cells.

The primary aim was to obtain quantitative information on the kinetics of sGC activation, deactivation, and desensitization, and the results are summarized in schematic form in Fig. 6*a*. A model analogous to ones developed to describe the binding and gating of neurotransmitter receptors (20) is depicted in Fig. 6*b*.

The activation kinetics (predicted to be second order) was too rapid to be resolved even with a 20-ms sampling time (at 30 μM caged-NO). No delay between uncaging of NO and the start of cGMP synthesis was detectable, although the data could accommodate a lag of a few milliseconds. This lack of detectable delay also applied to lower NO concentrations (see Fig. 5*c*). From studies of purified sGC (21–23), there appears to be general agreement that the NO-free state of sGC heme is a 5-coordinate ferrous (Fe^{2+}) center, with a proximal bond to the histidine residue at position 105 of the β-subunit. Binding of NO, creating the 6-coordinate form of the enzyme, is extremely rapid (near diffusion limited) and is followed by a transition to the catalytically active 5-coordinate species as a result of cleavage of the histidine bond (Fig. 6*b*). This step is considered to be rate-limiting. One report has concluded that the 6- to 5-coordinate transition is NO concentration-dependent in that at substoichiometric levels of NO there is a pronounced lag in onset of catalytic activity (at 4 °C), which decreases at higher levels of NO (22). This has been interpreted as evidence for a second (nonheme) NO-binding site. With NO in excess over sGC, the lag was around 100 ms, and this would presumably be much less at physiological temperatures. Another report (21) indicated that the transition occurred exponentially with a rate constant of 38 s^{-1} (half-time = 18 ms) at 15 °C. One assumes that the half-time would reduce to a few milliseconds at 37 °C. Consequently, there is no indication from our data that the kinetics of activation of sGC determined using the purified enzyme in an artificial environment is not applicable to the enzyme inside cells.

The rate of deactivation, however, represents one parameter where there is a major divergence between the behavior of purified and cellular sGC. For reasons that are unclear, there is disagreement between different laboratories on the rate of deactivation of the purified enzyme. Kharitonov *et al.* (24) using Hb or myoglobin to remove NO estimated the half-life for loss of NO from nitrosyl-sGC was 2 min at 37 °C. When substrate (GTP) and cofactor (Mg^{2+}) were included, however, the half-life was greatly accelerated to an estimated 5 s at 37 °C. Brandish *et al.* (25), in contrast, obtained a 2.5–3-min half-life (at 37 °C) irrespective of the presence of GTP and Mg^{2+}. Margulis and Sitaramayya (26), on the other hand, reported from sGC activity measurements (with GTP and Mg^{2+} present) a deactivation half-time of 18 s (20 °C), extrapolated to 5 s at 37 °C. Our experiments indicate that sGC deactivation in cells at 37 °C is much faster and does not occur in a kinetically straightforward manner. The data could be described operationally as an exponential decline, with a rate constant of 3.7 s^{-1}. Strictly speaking, this constant describes the rate at which sGC catalytic activity declines to zero, and this may not just reflect deactivation if progressive desensitization also contributes to the decline in sGC activity. In this case, the rate constant for deactivation would be the measured rate constant (k = 3.7 s^{-1}) minus the rate constant for desensitization (k = 0.14 s^{-1}) over the 600-ms period, giving a slightly lower value of 3.56 s^{-1}. This correction, however, makes the assumption that desensitization would proceed as normal after removal of free NO. As NO appears to contribute to desensitization (see below), this assumption may not be valid. Consequently, we favor an operational constant of 3.7 s^{-1} as a description of the slowest rate at which sGC deactivates after removal of NO. This corresponds to a half-time of 190 ms, a value 25-fold faster than the fastest estimate made using purified sGC (24, 26).

Mechanistically, it is unclear exactly what the value is describing. In the model (Fig. 6*b*), the rate of deactivation would correspond to the rates of the "back" reactions for NO dissociation from sGC (*i.e.* the steps with the constants k_{-1} and k_{-2}) minus the rates of the "forward" reactions (k_1 and k_2), and the observed rate constant would correspond to the rate-limiting step; this is likely to be the transition from the active, 5-coordinated species to the 6-coordinated species (k_{-2}; Ref. 22). Alternatively (or in addition), NO could dissociate directly from the 5-coordinate state without passing through the 6-coordinate intermediate, and the heme would then presumably revert to the resting, 5-coordinate, histidine-bound form; if so, this is to what the rate constant would correspond. Either way, the results imply that factors exist within cells that enable sGC to deactivate much faster than has so far been observed for the purified enzyme.

Desensitization of sGC has not been observed with the purified enzyme but was first identified in a previous study on cellular sGC (11). In the present investigation, the falling off of sGC activity with time could be adequately described with a single exponential. A desensitization time constant of 6.9 s was found when the NO concentration was one giving maximal

sGC activity after 1 or 10 s, but at a 10-fold higher concentration, the value decreased, indicating that NO accelerates desensitization. There was a hint from the earliest time points that a much more rapid phase of desensitization also exists, but realistically, different methods are needed to probe these very early kinetics accurately. At present, very little is known of the mechanism of desensitization, and in principle, there are several possible routes to the desensitized state of sGC (Fig. 6*b*). It has to be assumed that cells are equipped with a factor that interacts with sGC to bring about this modification of activity. Two of the simpler hypotheses are as follows: (*a*) that the factor(s) compromises the catalysis of cGMP synthesis without interfering with NO binding and sGC activation; (*b*) that the factor interferes with the mechanism of NO binding, dissociation, or enzyme activation, thereby "locking" sGC in a low activity state.

One consequence of the acceleration of sGC desensitization by NO is that the concentration-response curve becomes bell-shaped with time. At the shortest exposure examined (100 ms), when sGC will be close to nondesensitized, the apparent EC_{50} for NO was about 45 nM. This is 5-fold lower than the EC_{50} value estimated for the nondesensitizing purified enzyme (27) but is similar to the value measurable from other published data (28, 29). With the onset of desensitization (1 or 10 s of exposure), however, the apparent EC_{50} fell to about 20 nM. For the 10-s time point, this value should be considered an upper limit because the half-life of NO in the cell suspension is 10 s or less.[2] The reduction in EC_{50} with time may, in part at least, simply reflect the maximal rate of activation becoming truncated by desensitization (*cf.* Fig. 5*b*), although it is also possible that the desensitized form has a higher affinity for NO or that the efficacy for transition to the desensitized state is greater than for transition to the nondesensitized state. A method for maintaining NO at a constant concentration will be needed to understand the kinetics of desensitization in further detail.

Functionally, the kinetic properties of sGC will play an important role in decoding NO signals. First, the minimal delay between the arrival of NO and the generation of cGMP (at most a few ms) means that sGC should be capable of registering transient pulses of NO almost contemporaneously with their generation. In neurons, NO synthase is activated by Ca^{2+}/calmodulin, typically as a result of Ca^{2+} influx through ion channels associated with the *N*-methyl-D-aspartate subtype of glutamate receptor, to which the NO synthase is tethered via postsynaptic density proteins (2). Following release of a single quantum of glutamate, *N*-methyl-D-aspartate receptor channel opening leads to an elevation of cytosolic Ca^{2+} lasting for periods in the 100-ms range (30), and so a minimal NO pulse should have a similar duration. Such a pulse, over the same time scale, should be able to diffuse 10s of micrometers (31) to access sGC in neighboring cells and then activate the enzyme with negligible delay. The kinetic matching between NO generation and the activation of sGC located at a distance should therefore provide the receptive cell with temporal information about NO generation.

The rate of deactivation of sGC when the NO signal disappears is another key parameter governing the dynamics of the signaling pathway, and again, our finding that the enzyme becomes inactive in the 100-ms time scale indicates a kinetic fit to other properties of the pathway, although the dynamics of NO itself *in vivo* are still unclear. Hence, sGC activity is unlikely to retain a significant "memory" for NO as would be the case if deactivation took the several minutes suggested by some of the data on the purified enzyme (25).

Finally, during more prolonged periods of NO formation the transition, over a time scale of seconds, of sGC to its desensitized state may serve two functions. First, it would conserve GTP once time has been allowed for the cGMP concentration to rise. An analogy here is the way one heats up soup, using full heat to begin with and then turning it down as the desired temperature is approached. Second, in cells with different phosphodiesterase activities, desensitization permits diverse patterns of cGMP responses, ranging from brief transients to prolonged plateaus (11). Given the limited molecular heterogeneity of sGC and NO synthase, this may be a major way that diversity is introduced into the NO signaling pathway.

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[2] C. H. Griffiths and J. Garthwaite, unpublished observations.

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