Rate of Deactivation of Nitric Oxide-Stimulated Soluble Guanylate Cyclase: Influence of Nitric Oxide Scavengers and Calcium†

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ABSTRACT: Soluble guanylate cyclase (sGC) is highly activated by nitric oxide (NO) and is the known mediator of the effects of NO on a variety of physiological processes. The rates at which sGC is activated and deactivated are therefore of wide interest since they determine the duration of a tissue's response to NO. The effect of NO on smooth muscle dissipates in 1−2 min, suggesting that both activation and deactivation are fast. In vitro measurements show that the activation of sGC occurs in less than a second, while the deactivation takes several hours at 20 °C. However, recent reports indicate that Mg-GTP, oxyhemoglobin, and reducing and oxidizing agents could deactivate the cyclase in several seconds to minutes, though the effectiveness of each of these agents is in dispute. We investigated the lifetime of NO-sGC in the cytosol of retina by monitoring its enzymatic activity at 20 °C. Our results show that Mg-GTP, the substrate of NO-sGC, has no influence on the deactivation. Similarly, reducing agents glutathione and dithiothreitol shortened the half-life of NO-sGC only by about 30%. The greatest effect on the deactivation was caused by scavengers of NO: oxyhemoglobin reduced the half-life of NO-sGC from 106 min to 18 s; another NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), reduced it to 42 s (20 °C). Similarly rapid deactivation was observed with the enzyme from bovine lung, immunoprecipitated enzyme from bovine retina, and heme-deficient enzyme from bovine retina reconstituted with heme. On the other hand, YC-1, an activator of sGC, stabilized the activated enzyme, preventing NO dissociation, as was evident from the inability of oxyhemoglobin or CPTIO to deactivate NO-sGC. Calcium, which is known to inhibit NO-sGC, also inhibited the effects of oxyhemoglobin and CPTIO, slowing down the deactivation of the enzyme. Lithium, which is also known to inhibit NO-sGC, had no effect on the deactivation rate of the enzyme. These results, taken together, suggest that two factors with major impact on the lifetime of NO-sGC are the proximity to NO scavengers and the calcium concentration in the cell.

Nitric oxide (NO)[1] plays an important role in vasodilatation and neuronal signal transduction through the mediation of soluble guanylate cyclase (sGC), the only known physiological receptor of NO (*1*−*4*). The rates of activation of sGC by NO and of the subsequent deactivation determine the duration of response to NO and are, therefore, of particular interest. Soluble GC is a heterodimer, consisting of α and β subunits, with ferrous heme attached to the β subunit (*5*−*8*). Upon binding of NO, sGC is activated as much as 670-fold (*9*). NO has an unusually high binding affinity for heme [equilibrium constant of about 10^{15} M^{-1} (*10*)], but the activation of cyclase by NO is a relatively slow process (*11*). It is shown that in the unactivated enzyme heme is bound to a histidine residue in the β subunit (*7*, *8*, *12*). The association of NO with the heme iron breaks the trans bond between histidine and iron, thereby activating the enzyme. This break is the rate-limiting step (0.1−1.0 s^{-1}), contributing to the slowness of the overall activation process despite the high affinity of NO for heme (*11*). The suggestion that the breakage of the histidine−heme bond leads to activation of the enzyme is based on the observation that metal-free protoporphyrin can activate sGC to the same level as NO (*13*). Deactivation of NO-stimulated sGC (NO-sGC) is thought to occur by dissociation of NO from the activated enzyme (*9*, *14*). The biological response of smooth muscle to NO, which is mediated by cGMP, dissipates in about 1−2 min (*15*). Since the recovery of the muscle from NO-induced relaxation would depend on not only the termination of NO-sGC activity but also the downstream reactions initiated by cGMP, it is reasonable to assume that NO-sGC deactivates in much less than 1−2 min.

Recent in vitro studies have shown that NO-sGC remains active for much longer than 1−2 min but that its deactivation is accelerated by oxyhemoglobin (scavenger of NO) (*9*), Mg-GTP (substrate of sGC) (*16*), or redox agents (*9*, *17*).

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[1] Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; dibromo-BAPTA, 5,5′-dibromo-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid]; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GSH, reduced glutathione; GTP, guanosine 5′-triphosphate; HbO_2, oxyhemoglobin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NO, nitric oxide; PDE, phosphodiesterase; PMSF, phenylmethanesulfonyl fluoride; sGC, soluble guanylate cyclase; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole.

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Kharitonov et al. (*16*) found that Mg-GTP greatly reduces the half-life of NO-sGC, making it short enough to account for the deactivation rate of the enzyme in the biological systems. However, Brandish et al. (*9*) did not observe any effect of Mg-GTP on the deactivation rate under similar conditions. The latter researchers reported that the deactivation is accelerated more than 30-fold by oxyhemoglobin, but even so, it is not fast enough to account for the rate of deactivation in vivo. Therefore, the roles of Mg-GTP and oxyhemoglobin in regulating the deactivation of NO-sGC remain uncertain. The influence of redox agents on the deactivation of NO-sGC also needs to be resolved, with both reducing and oxidizing agents reported to accelerate it (*9*, *17*). The mechanism of deactivation of NO-sGC under aerobic conditions is not well understood: reports indicate that the enzyme is either dissociated into NO and ferrous sGC or partially oxidized to ferric sGC and nitrate (*9*, *17*). Thus, the mechanism and the rate of deactivation of NO-sGC still remain to be clarified and form the focus of the present work.

We investigated the lifetime of NO-sGC in the cytosol of retina by directly monitoring its enzymatic activity. We found that the deactivation of NO-sGC was greatly accelerated by the NO scavengers oxyhemoglobin (HbO_2) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO). The half-life of NO-sGC, measured at 20 °C, was reduced from about 106 min in the absence of scavengers to about 18 and 42 s in the presence of HbO_2 and CPTIO, respectively (extrapolated to 37 °C, the half-lives in the presence of HbO_2 and CPTIO are 5 and 13 s, respectively). Reducing agents, dithiothreitol (DTT) and glutathione (GSH), did not significantly affect the rate of NO-sGC deactivation. In contrast, the NO-independent sGC activator YC-1 and Ca^{2+}, which is known to inhibit NO-sGC, both markedly prolonged the lifetime of NO-sGC.

MATERIALS AND METHODS

Preparation of Retinal Cytosol. Fresh bovine eyes were obtained from a local slaughterhouse and brought to the laboratory on ice. All subsequent operations were conducted under room light at 4 °C. Cytosol was prepared as described earlier (*18*). Briefly, retinas were removed and placed in tubes (3 retinas/tube) containing 10 mL of ice-cold buffer A (0.32 M sucrose and 5 mM Tris-HCl, pH 7.4). Rod outer segments were partially removed by gentle vortexing for 5 s and aspirating the buffer. After three repetitions of this procedure, each with the addition of 10 mL of buffer A, retinas were homogenized (5 strokes in glass/Teflon homogenizer) in 10 mL/tube buffer A supplemented with 4 mM isobutylmethylxanthine (IBMX) and protease inhibitors (10 μg/mL each aprotinin, leupeptin, and trypsin inhibitor, and 50 μg/mL benzamidine). The homogenate was centrifuged at 150*g* for 10 min to remove the nuclei and cell debris, and the supernatant was respun. The resulting supernatant was centrifuged first at 800*g* for 10 min and then at 25000*g* for 12 min. The final supernatant was aliquoted and frozen at −70 °C. On the day of the experiment an aliquot was thawed and centrifuged at 100000g for 1 h, and the supernatant (cytosol) was used in the guanylate cyclase assay.

Preparation of the Heme-Deficient and Heme-Reconstituted sGC. Heme-deficient, partially purified sGC was prepared according to Ignarro et al. (*19*) with a slight modification. Briefly, 25 frozen retinas were homogenized (20 strokes in glass/Teflon homogenizer) in 50 mL of buffer B (25 mM triethanolamine hydrochloride, pH 7.8, containing 5 mM DTT, 0.1 mM PMSF, and protease inhibitors). Following two subsequent low-speed centrifugations (10000*g* for 10 min), the resulting supernatant was further centrifuged at 100000*g* for 1 h, and the supernatant was recentrifuged to obtain the soluble fraction. An equal volume of a saturated ammonium sulfate solution was added to this fraction and the precipitated protein was collected. The precipitate was dissolved in 5 mL of buffer B and dialyzed extensively against the same buffer. The sample (approximately 7.3 mL after removal of any insoluble material by centrifugation at 100000*g* for 30 min) was applied on a 1 × 5 cm column of DE52 cellulose, previously equilibrated with buffer B. The effluent was cycled back twice, and the column was washed with 50 mL of buffer B and eluted with 50 mL of a linear NaCl gradient (0−0.5 M) in the same buffer. Two-milliliter fractions were collected and analyzed for guanylate cyclase activity in the presence and absence of 0.1 mM sodium nitroprusside, and those with high basal and low stimulated activity were pooled, supplemented with 10 mM DTT and 0.1 mM PMSF, and stored at 4 °C (heme-deficient sGC). Heme-restored sGC was obtained by adding hematin to the heme-deficient enzyme containing DTT (*19*).

Preparation of NO-sGC. All manipulations were on ice, unless otherwise indicated. The nitrosyl adduct of cytosolic sGC was prepared anaerobically as follows: A sample of cytosol was deaerated in a 4 mL stirred rubber-sealed vial by a conventional gas train with five vacuum/nitrogen cycles, with a 1 min of equilibration between cycles. A second rubber-sealed vial, containing buffer C (buffer A with 4 mM IBMX), was deaerated as above. A saturated solution of NO gas was prepared by bubbling NO (passing through a 50% w/w solution of KOH) through deaerated buffer C for 5 min, followed by 15 min of equilibration. A gastight syringe, flushed four times with the saturated NO solution, was used to inject an aliquot of the same solution into a vial containing the deaerated cytosol, to bring the calculated concentration of NO to 10 μM. A Pharmacia PD-10 desalting column was used to remove excess NO (not bound to sGC), as described by Brandish et al. (*9*).

Guanylate Cyclase Assay. Every batch of cytosol was checked for phosphodiesterase (PDE) activity by including 3 nCi/μL [8-^{3}H]cyclic GMP in the assay. Only preparations with no detectable hydrolysis of cyclic GMP during the assay were used in the following studies.

To study the deactivation rate of NO-sGC, the following experimental design was employed: 600 μL of NO-sGC were placed in a 1 mL cylindrical thermostated (20 °C) cuvette, equipped with a stir bar. After 5 min of temperature equilibration, the reaction was initiated by the addition of 200 μL of a substrate mixture preequilibrated to 20 °C. The final concentration of reagents in the assay mixture was as follows: 40 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 0.5 mM GTP, 2 mM cGMP, 25 μM zaprinast, 15 mM phosphocreatine, 0.2 mg/mL creatine phosphokinase, 2 mM EGTA, 2.5 mM IBMX, and 0.3 μCi/μL [α-^{32}P]GTP. At desired time intervals after the reaction was started, 40 μL aliquots were withdrawn and placed in Eppendorf tubes containing 20 μL of a stop solution (150 mM EDTA supplemented with 2 mM

each cyclic GMP, GMP, and GTP). The tubes were heated at 95 °C for 5 min, cooled, and centrifuged. The [^{32}P]cyclic GMP formed in the assay was separated by TLC and measured as described earlier (*20*). The specific activity of NO-sGC varied between preparations and was usually in the range of 1.2−1.8 nmol min^{-1} (mg of protein)$^{-1}$ at 20 °C (120−175-fold activation over the basal activity). During prolonged assays, as in Figure 2, a maximum of 20% of the substrate in the assay mixture was utilized.

In extrapolating from 20 to 37 °C, the deactivation rate of NO-sGC was assumed to double every 10 °C.

To investigate the effect of calcium, dibromo-BAPTA-buffered calcium solutions were substituted for EGTA in the assay mixture. Free calcium concentrations were calculated by using Maxchelator and verified by Corning calcium electrode.

In experiments where NO scavengers (HbO_2 or CPTIO) were used, a small volume of the respective stock solution was added to the cuvette 3−4 min after the reaction was started with the substrate. After sGC activity reached a plateau, a stock solution of NO was added to the cuvette in excess of scavenger to fully reactivate the enzyme. Enzyme activity was sampled further for 2−3 min.

In the experiments with heme-reconstituted or immunoprecipitated enzyme and the bovine lung extract, NO-activated enzyme was used in the assay without removal of excess NO. In these cases, the effect of oxyhemoglobin was investigated by adding it to the cuvette in excess of the NO present.

Immunoprecipitation of sGC from Retinal Extract. Retinal extract was prepared by homogenization (20 strokes, glass/Teflon homogenizer) of 6 fresh bovine retinas in 12 mL of buffer D (50 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM Mg^{2+}, 0.1 mM EDTA, and protease inhibitors). The homogenate was centrifuged for 10 min at 10000*g*, and the supernatant was respun. The resulting supernatant was centrifuged twice for 1 h at 100000*g*, aliquoted, and frozen at −70 °C. When used, an aliquot was thawed out, adjusted to 2 mM EDTA, 4 mM EGTA, 2 mM DTT, 0.2 mM PMSF, and 0.2 mM sodium vanadate, and incubated for 1 h at room temperature and then overnight at 4 °C with 0.1 volume of sGC antiserum. The immunoprecipitation mixture was then rotated for 2 h at room temperature with protein A−agarose washed in buffer E (40 mM HEPES, pH 7.4, 40 mM NaCl, 2 mM EDTA, and 2 mM DTT). After absorption, the resin was washed four times with and suspended in buffer E. This suspension was used for the deactivation rate measurement as described above.

Bovine Lung Extract Preparation. Fifty grams of fresh tissue was cleaned, cut into small pieces with a razor blade, rinsed with buffer D, minced in a blender, and finally homogenized in an equal volume of buffer D. The homogenate was spun for 10 min at 10000*g*, the supernatant was respun, and the final supernatant was spun for 1 h at 100000*g*, aliquoted, and frozen at −70 °C.

Oxyhemoglobin Preparation. Oxyhemoglobin was prepared by adding a 10-fold molar excess of sodium dithionite to a 2 mM solution of hemoglobin in 100 mM HEPES, pH 7.4, followed by saturation of the mixture with the oxygen gas. Oxyhemoglobin concentration was determined spectrophotometrically (*21*) and adjusted to 2 mM heme. The resulting stock solution was aliquoted and frozen at −70 °C.

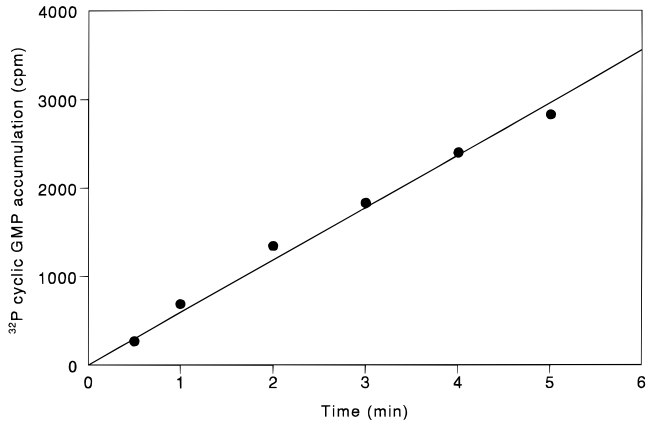

FIGURE 1: Rate of cGMP production by NO-sGC in the presence of Mg-GTP. Enzyme activity was assayed as described under Materials and Methods. Data shown are from one of six experiments with similar results.

Excess sodium dithionite was not removed from the stock solution since it was found in the preliminary experiments that up to 1 mM sodium dithionite in the assay had no effect on basal or NO-stimulated activity or on the deactivation rate.

Materials. Bovine eyes and lungs were obtained from Wolverine Packing Company, Detroit, MI. [α-^{32}P]GTP was from NEN Life Science Products, Inc., Boston, MA. [8-^{3}H]-Cyclic GMP was from ICN Pharmaceuticals, Inc., Irvine, CA. CPTIO was purchased from Calbiochem−Novabiochem Corp., La Jolla, CA. Protein A, immobilized on agarose, was from Pierce, Rockford, IL, and DE52 cellulose was from Whatman International Ltd., Maidstone, England. Polyclonal sGC antibody was purchased from Alexis Biochemicals, San Diego, CA. Gases (N_2, NO, and O_2) were obtained from AGA Gas, Inc., Bloomfield Hills, MI. All other reagents were from Sigma Chemical Co., St. Louis, MO.

RESULTS AND DISCUSSION

Stability of NO-sGC in the Presence of Mg-GTP, GSH, and DTT. In a recent study, Kharitonov et al. (*16*) observed that Mg-GTP, the substrate of sGC, accelerates the deactivation of NO-sGC. Measuring the deactivation rate of NO-sGC by following the interaction of the released NO with oxyhemoglobin, they reported that Mg-GTP accelerates the deactivation nearly 50-fold, reducing the half-life ($t_{1/2}$) from 2−4 min to 5 s at 37 °C. Since we measured the deactivation of NO-sGC by following its enzymatic activity, Mg-GTP was an unavoidable constituent of our assay mixture. It is therefore impossible to compare the deactivation rate in the presence and absence of this test agent. However, if Mg-GTP deactivates NO-sGC in seconds, the effect should become evident in a time-course measurement of the activated enzyme. As shown in Figure 1, the rate of cGMP production by NO-sGC was linear for several minutes. Actually, there was no significant loss of activity within the first 15 min of assay at 20 °C (data not shown). Therefore, Mg-GTP appeared not to influence the deactivation of NO-sGC, at least for the duration of the experiment.

Our experiments were done with unpurified NO-activated sGC in the cytosol of retina homogenate, while Kharitonov et al. (*16*) used an NO-activated purified lung enzyme in their studies. The lack of effect of Mg-GTP in our study

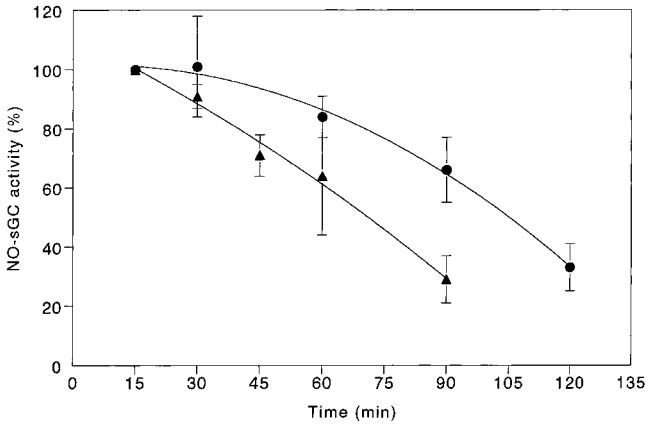

FIGURE 2: Influence of glutathione on the deactivation of NO-sGC. Accumulation of cGMP was monitored in the presence (▲) or absence (●, control) of 1 mM GSH. Activity for each time point was calculated as the amount of cGMP, accumulated during the interval from the preceding time point, divided by the length of the interval, and shown as percent of activity at the beginning of the experiment. The measurements were done in triplicate; each point represents the mean of three different experiments, and the error bars indicate SD. Similar results were obtained with 2 mM DTT.

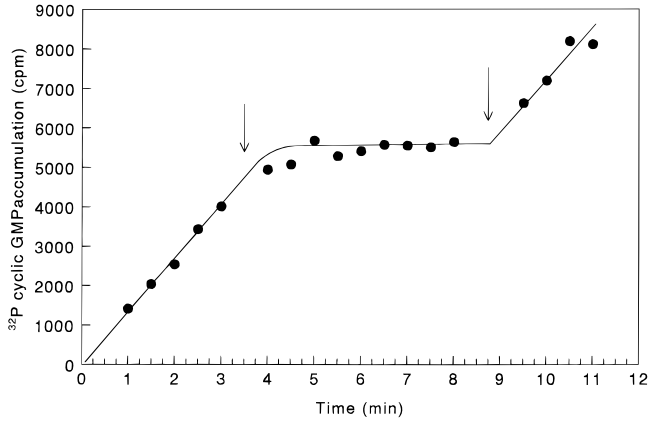

FIGURE 3: Rapid, reversible deactivation of NO-sGC by oxyhemoglobin. Cyclase reaction was initiated with Mg-GTP at time zero as described under Materials and Methods. At the time point indicated by the left arrow, 6 μL of a 2 mM stock solution of HbO_2 was added to the cuvette (20 μM final concentration). At the time point indicated by the right arrow, 5 μL of a saturated NO solution was added to the cuvette (40 μM final concentration). The data presented here are from one of seven different experiments, from which the half-life is calculated to be 18 ± 2 s.

could have been due to the presence of other proteins in the assay. However, we note that other investigators who worked with purified enzyme, purified from bovine lung by either conventional (*11*) or immunoaffinity method (*23*), found its NO-stimulated activity to be linear with incubation time. Therefore, proteins other than sGC could not have been responsible for the lack of effect of Mg-GTP in our experiments on retina cytosol. In addition, other NO-activated sGC preparations, such as the enzyme in bovine lung cytosol, immunoprecipitated sGC from retina cytosol, and a partially purified enzyme from retina cytosol, all exhibited a linear time course. We therefore conclude that Mg-GTP does not have a significant influence on the deactivation of NO-sGC.

Glutathione and DTT were reported to accelerate the deactivation of NO-sGC more than 10-fold, reducing the half-life from 87 to 6.3 and 4.3 min, respectively, at 37 °C (*9*). In our experiments, however, the effect of reducing agents was marginal: the half-life of NO-sGC was 106 min in the control and 73 min in the presence of 1 mM glutathione (20 °C), a reduction of 31% (Figure 2). Extrapolated to 37 °C, the half-lives would correspond to 32 and 22 min, respectively. The effect of DTT was similar (data not shown).

Kharitonov et al. (*16*) reported that addition of Mg-GTP to the assay containing oxyhemoglobin and GSH or DTT (which were obligatory components of their assay) reduced the half-life of NO-sGC from 2−4 min to 5 s. Brandish et al. (*9*) found that oxyhemoglobin reduced the half-life of NO-sGC from 6.3 to 2.9 min in the presence of GSH. However, they noted that addition of Mg-GTP did not further accelerate the deactivation. The difference between these two reports on the combined effect of Mg-GTP and oxyhemoglobin on the deactivation of NO-sGC, and our observation described above, that Mg-GTP alone has no effect, prompted us to investigate the role of HbO_2.

Effect of Oxyhemoglobin on the Deactivation Rate. To test its effect on the deactivation rate, we added HbO_2 to the reaction mixture simultaneously with the substrate Mg-GTP, as we did with GSH and DTT in the experiments described above, and sampled the activity over several minutes. However, there was no measurable cGMP accumulation in the very first aliquot sampled at 5 min after initiation of the reaction. This observation suggested that oxyhemoglobin must have deactivated the enzyme very rapidly. We therefore switched to the assay design where HbO_2 was added to the assay mixture 3−4 min after the reaction was initiated with substrate, and the activity was then sampled at shorter time intervals (see description in Materials and Methods). As shown in Figure 3, NO-sGC activity was linear before the addition of HbO_2. Addition of 20 μM HbO_2 to the cuvette completely abolished cGMP production, the activity decaying with a $t_{1/2}$ of 18 s. Extrapolated to 37 °C, this corresponds to a half-life of about 5 s, which is identical with the observation made by Kharitonov et al. (*16*) on incubations containing both Mg-GTP and HbO_2. Addition of NO to the deactivated enzyme, in excess of HbO_2, completely restored cGMP production to the pre-HbO_2 rate. This shows that NO-sGC was reversibly deactivated, not inactivated, by HbO_2.

From the above experiments, it appears that a rapid deactivation of NO-sGC is caused neither by the Mg-GTP nor reducing agents (GSH or DTT) but by the NO scavenger (HbO_2). If that is the case, other NO scavengers with greater affinity to NO than sGC should also cause rapid deactivation of NO-sGC. To test this possibility, we employed another NO scavenger, carboxy-PTIO (CPTIO). Figure 4 shows that CPTIO deactivated the NO-sGC, though about 2 times more slowly than HbO_2, reducing the $t_{1/2}$ to about 42 s (20 °C). The difference between the effectiveness of HbO_2 and CPTIO could probably be explained by the observation that hemoglobin reacts with NO about 3 times faster than CPTIO (*22*). The finding that two structurally different NO scavengers could both rapidly deactivate NO-sGC shows that the mechanism of deactivation is based on scavenging NO dissociated from NO-sGC.

Two mechanisms by which NO-sGC gradually loses activity under aerobic conditions were reported. Brandish et al. (*9*), working with an enzyme preparation isolated with a stoichiometric equivalent of heme, found the enzyme to

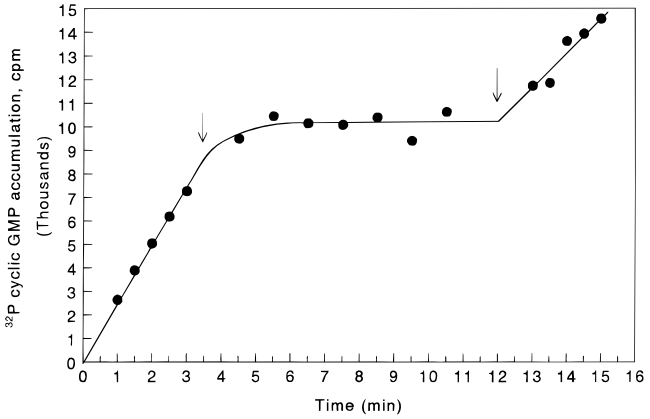

FIGURE 4: Deactivation of NO-sGC by CPTIO. Cyclase reaction was initiated as described under Materials and Methods. At the time point indicated by the left arrow, 85.7 μL of a 3.6 mM stock solution of CPTIO was added to the cuvette (450 μM final concentration). At the time point indicated by the right arrow, 25 μL of a saturated NO solution was added to the cuvette (128 μM final concentration). Correction was made in the measured counts per minute for the dilution caused by the addition of reagents to the incubation.

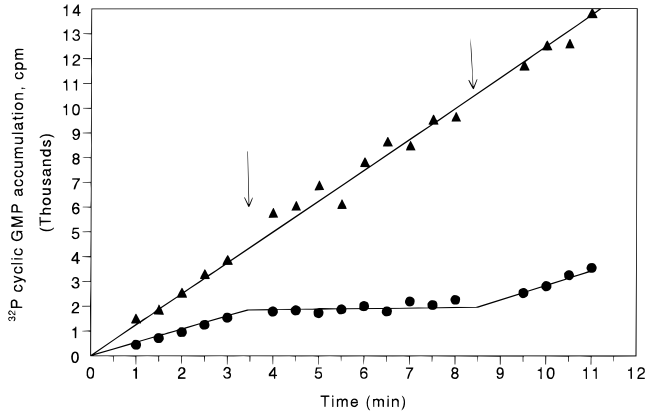

FIGURE 5: Influence of YC-1 on the activity and deactivation of NO-sGC. Accumulation of cGMP in the assay mixture was monitored in the absence (●, control) or the presence (▲) of 30 μM YC-1. At the time point indicated by the left arrow, 18 μL of a 2 mM stock solution of HbO_2 was added to the cuvette (60 μM final concentration). At the time point indicated by the right arrow, 20 μL of a saturated NO solution was added to the cuvette (160 μM final concentration). Correction was made in the measured counts per minute for the dilution caused by the addition of reagents to the incubation.

dissociate into NO and ferrous-sGC. Dierks and Burstyn (*17*), who used a heme-deficient sGC reconstituted with heme and activated by NO, found that deactivation of the enzyme is associated with formation of ferric sGC and nitrate. There could have been a difference in the heme environment of these two preparations contributing to the dissimilar behavior of the enzymes (*9*). If such a difference exists, it might be reflected in the rates of deactivation of the enzymes also. To test this possibility we prepared heme-deficient enzyme, reconstituted it with heme, and activated with NO as described under the Materials and Methods. Upon addition of oxyhemoglobin, this enzyme deactivated at a rate similar to that of the native enzyme (half-life of about 18 s at 20 °C; data not shown). Therefore, we concluded that if there is a difference between native and heme-reconstituted enzymes in their heme environment, it had no influence on the dissociation rate of NO. Subsequent addition of NO to the deactivated enzyme, whether native or heme-reconstituted, fully restored the activity, suggesting that the mechanism of deactivation of both enzymes is similar, through dissociation to NO and ferrous sGC.

YC-1 Slows Down the Deactivation Rate. Friebe and Koesling (*23*) observed that in the presence of YC-1, an NO-independent activator of sGC, oxyhemoglobin was less effective in inhibiting NO-sGC. They concluded that YC-1 stabilized the activated configuration of NO-sGC and diminished the dissociation of NO from the enzyme. However, the concentration of YC-1 used in their experiments (200 μM) was more than 3 times higher than the concentration of HbO_2. It is therefore unclear whether the observed effect is due to interaction of YC-1 with NO-sGC and its stabilization, or due to the interaction of YC-1 with HbO_2, reducing the latter's ability to scavenge NO. One way of addressing this question is to test if YC-1 slows down the deactivation when oxyhemoglobin is in excess. We tested the ability of 60 μM HbO_2 to deactivate the enzyme in the presence of 30 μM YC-1. As shown in Figure 5, YC-1 completely prevented deactivation of NO-sGC for the duration of the experiment. This observation suggests that the actions of YC-1 and oxyhemoglobin are independent: oxyhemoglobin removes dissociated NO, while YC-1 prevents the dissociation of NO, as suggested by Friebe and Koesling (*23*). In addition to the effect on dissociation of NO, YC-1 also potentiated the activity of fully activated NO-sGC, in agreement with previous reports (*23*, *24*).

Ca^{2+} Slows the Deactivation Rate of NO-sGC. Effects of Ca^{2+} on basal and stimulated activities of sGC were investigated extensively, yielding inconclusive results: Steuer and Schütz (*25*) showed that Ca^{2+} at 0.2 μM and higher concentrations inhibits NO-stimulated sGC, whereas MacNeil found that increasing the Ca^{2+} concentration from 10 nM to 1 μM activates sGC. Mayer et al. (*27*) suggested that the Ca^{2+}-dependent decrease in cGMP concentration in the cytosol of synaptosomes is due to activation of cGMP phosphodiesterase rather than to inhibition of sGC. However, recent studies with purified or immunoprecipitated sGC indicate that Ca^{2+} directly inhibits NO-stimulated activity of sGC (*9*, *28*). We argued that the observed inhibition could be due to either direct inhibition of the enzyme or due to acceleration of its deactivation. To investigate this question we measured the activity and deactivation of NO-sGC at different free Ca^{2+} concentrations. The results, presented in Figure 6, show that calcium inhibits NO-sGC. To our surprise, however, we found that calcium also slows down the deactivation of NO-sGC in a dose-dependent manner. At 10 μM calcium, the activity was inhibited by 80%, and the half-life of NO-sGC was increased from about 18 s to 72 $\pm$ 5 s (n = 3; 20 °C). The influence of calcium on the deactivation rate could result from its interaction with NO-sGC or HbO_2. Since the concentration of HbO_2 was higher than that of free calcium and similar effects of calcium were observed when CPTIO (450 μM) was used in place of HbO_2 (data not shown), we conclude that calcium interacts with NO-sGC. However, whether the interaction is directly with the enzyme or through a regulatory calcium-binding protein remains to be investigated.

Lithium is another cation known to inhibit NO-sGC (*29*, *30*). To determine whether lithium also has dual effects on the enzyme, we measured the activity and deactivation of

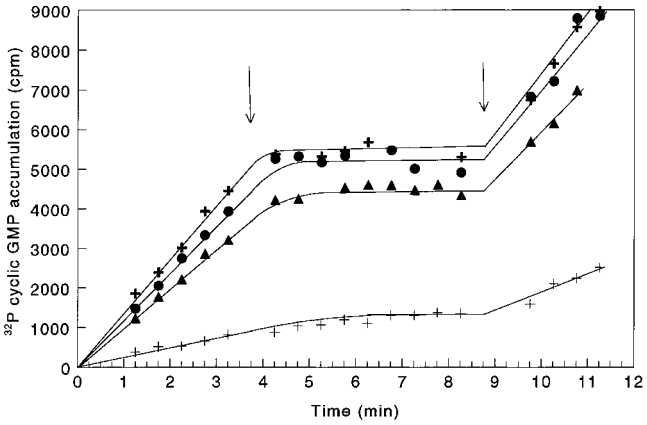

FIGURE 6: Effect of calcium on the activity and deactivation of NO-sGC. NO-sGC was assayed with different calcium concentrations in the reaction mixture as described under Materials and Methods. Calcium concentrations: (bold +) 60 nM; (●) 485 nM; (▲) 918 nM; (+) 9.7 μM. At the time point indicated by the left arrow, 6 μL of a 2 mM stock solution of HbO_2 was added to the cuvette (20 μM final concentration). At the time point indicated by the right arrow, 5 μL of a saturated NO solution was added to the cuvette (40 μM final concentration).

NO-sGC in the presence of 50 mM LiCl. Lithium inhibited the enzyme, as expected, but had no effect on the deactivation rate (data not shown). This observation suggests that inhibition of NO-stimulated enzyme does not necessarily affect the deactivation rate and indicates that retarding the deactivation may be an effect specific to calcium.

The prolonged activation of sGC in the presence of calcium, though at a much reduced level, could be due to production of small amounts of NO in the assay mixture. However, this is unlikely to be the case in our experiments because the NO-activated enzyme preparation was passed through a PD-10 column before measurement of the deactivation rate, which removes not only free NO but also other small molecules such as arginine and NADPH required for NO production. Coupled with the observation that lithium did not prolong the activation of a similarly prepared enzyme, it is likely that the effect of calcium is specific and not due to continued low-level production of NO.

CONCLUSIONS

The observations made in this report show that neither Mg-GTP nor reducing agents have a significant effect on the lifetime of NO-activated sGC. NO scavengers, however, are capable of causing full and reversible deactivation of NO-sGC in seconds. This suggests that NO is in equilibrium between free and sGC-bound forms and that the availability of scavengers in the vicinity with greater affinity to NO than sGC permits rapid deactivation of the enzyme. We also show that calcium, a known inhibitor of NO-sGC, paradoxically increases its lifetime. A rise in the cellular calcium concentration, therefore, has the effect of causing a prolonged, low-level synthesis of cyclic GMP in response to NO.

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