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ACCELERATED COMMUNICATION

Characterization of 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one as a Heme-Site Inhibitor of Nitric Oxide-Sensitive Guanylyl Cyclase

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SUMMARY

Nitric oxide (NO) binds with high affinity to the heme of soluble guanylyl cyclase (sGC), resulting in accumulation of the second messenger cGMP in many biological systems. 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) was recently described as potent and selective inhibitor of sGC, providing an invaluable tool with which to settle the function of the cGMP pathway in NO-mediated signal transduction [*Mol. Pharmacol.* **48**:184–188 (1995)]. The present study investigated the mechanism of ODQ-induced inhibition of purified bovine lung sGC. The drug induced a rightward shift of the concentration-response curves recorded with two different NO donors and a reduction of maximal sGC activity, pointing to a mixed type of inhibition. The time course of NO-stimulated sGC activity determined in the presence of 0.3 μM ODQ showed that the inhibitory effect was time-dependent (half-time ~3 min) and virtually complete after about 10 min. The cyclase did not recover from ODQ-induced inhibition upon extensive dilution, pointing to an apparently irreversible inactivation of the enzyme by the quinoxalin. Light absorbance spectroscopy showed that ODQ (0.3 mM) induced a shift of the Soret band of the heme from 431 nm to 393 nm, indicating that ODQ oxidizes the ferrous form of the enzyme to the ferric species, which is thought to exhibit only poor NO sensitivity. Together, our results suggest that inhibition of sGC by ODQ is NO-competitive and results in an apparently irreversible oxidation of the prosthetic heme group.

NO regulates a variety of physiological processes, such as smooth muscle relaxation, platelet aggregation, and synaptic transmission in the brain (1, 2). Most of the physiological effects of NO are mediated through direct stimulation of sGC [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2.], which catalyzes the formation of cGMP from GTP with the concomitant release of pyrophosphate (3, 4). NO-sensitive sGC is a heterodimer composed of an α and a β subunit with calculated molecular masses of 77 and 70 kDa, respectively (5, 6). The enzyme was reported to contain stoichiometric amounts of ferroprotoporphyrin IX (7), which was shown to mediate NO stimulation of the enzyme (8). The heme group is linked to His105 of the β subunit (9) and resembles that of hemoglobin and myoglobin, but sGC exhibits unusual binding properties for small ligands such as cyanide or oxygen, presumably due to an extraordinary coordination environment (10, 11). As shown previously, the UV/VIS spectrum of native sGC features a sharp Soret peak at 431 nm and a single broad α/β peak at 555 nm, which is indicative for a five-coordinate high-spin ferrous heme structure with histidine as the axial ligand (10). However, other reports provide evidence that sGC contains predominantly low-spin bis-histidine-ligated heme in its resting state (12, 13). NO stimulation of sGC is due to high affinity binding of NO to the ferrous heme iron and consequent breaking of the bond between the metal center and the proximal histidine (14). Release of the imidazole ligand may lead to a change in heme geometry and protein conformation, resulting in enzyme activation. The formation by NO of a penta-coordinate ferrous nitrosyl-heme species is evident spectroscopically as a shift of the Soret peak from 431 to 398 nm, the appearance of a prominent

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ABBREVIATIONS: NO, nitric oxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine, GSNO, *S*-nitrosoglutathione; sGC, soluble guanylyl cyclase.

shoulder at 485 nm, and the splitting of the α/β region (10). The requirement of an enzyme-linked prosthetic heme group for NO-mediated activation of sGC is corroborated by the recent observation that site-directed mutagenesis of His105 of the β subunit yielded an enzyme that still exhibited basal activity but was insensitive to NO (9). Oxidizing agents like $K_3[Fe(CN)_6]$ convert the ferrous heme of sGC to the ferric form (7), which is thought to exhibit poor NO sensitivity. The UV/VIS spectrum of ferric sGC shows a Soret band at 393 nm (7, 10); the single broad α/β peak of the native enzyme at 555 nm is split into two peaks at 511 and 642 nm (15). Spin and coordination state of ferric sGC are still matters of controversy (10, 13).

Methylene blue and LY-83583 have been widely used as inhibitors of sGC to probe the involvement of the NO/cGMP signal-transducing pathway in various biological processes. However, both drugs have several other effects, including inhibition of NO synthase and superoxide-mediated inactivation of NO (16, 17). The quinoxalin derivative ODQ was recently described as a potent and highly selective inhibitor of sGC (18), making this compound an invaluable tool with which to discriminate between cGMP-dependent and -independent NO signaling (19, 20). Enzyme inhibition was reported to be noncompetitive with the substrate GTP (18), raising the question of how ODQ exerts its potent inhibitory effect. In the current study, we demonstrate that the drug binds in an NO-competitive manner to sGC, thereby oxidizing the prosthetic heme group to the ferric form.

Experimental Procedures

Materials. sGC was purified from bovine lung as described previously (21). For the experiments shown in Fig. 1C, the cDNAs encoding the wild-type (α_1/β_1) heterodimer and an NO-insensitive mutant (α_1/β_1/His105) were expressed in COS-7 cells as described previously (9). [α-^{32}P]GTP (400 Ci/mmol) was purchased from Med Pro (Amersham, Vienna, Austria). Solutions of DEA/NO (Research Biochemicals International, Natick, MA) were made with 10 mM NaOH. GSNO, a kind gift from Dr. Harold F. Hodson (Wellcome Research Laboratories, Beckenham, UK) was dissolved in 50 mM triethanolamine/HCl buffer, pH 7.4 [containing 0.5 mM [α-^{32}P]GTP (200,000–300,000 cpm), 3 mM $MgCl_2$, 1 mM cGMP, 0.5 mg/ml bovine serum albumin, and 1 mM GSH]. ODQ was a gift from Dr. E. B. Nielsen (Nova Nordisk, Denmark) the drug was dissolved in dimethylsulfoxide to a concentration of 100 mM and further diluted with 50 mM triethanolamine/HCl buffer, pH 7.4. All other chemicals were obtained from Sigma (Vienna, Austria).

Determination of sGC activity. Purified sGC (45 ng) was incubated at 37° for 10 min in a total volume of 0.1 ml of 50 mM triethanolamine/HCl buffer, pH 7.4. ODQ was present as indicated. Reactions were started by the addition of 10-fold concentrated stock solutions of DEA/NO, GSNO, or vehicle to the samples, followed by transfer of the samples from 0° to 37°. Reactions were stopped with $ZnCO_3$ precipitation, and [^{32}P]cGMP was isolated as described previously (22). Results were corrected for enzyme-deficient blanks and recovery of cGMP. For the determination of basal sGC activity, 0.2 μg of enzyme was incubated for 20 min under the conditions described above. Data represent mean values ± standard error of three experiments performed in duplicate. Parameters of the concentration-response curves were calculated according to the Hill equation.

In the preincubation experiments, 0.23 μg of sGC was incubated at 37° in 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.5 mg/ml bovine serum albumin and 1 mM GSH in the absence or presence of 0.3 μM ODQ. At the indicated time points, aliquots were removed, diluted 10-fold with the assay mixture described above, and further incubated for 10 min at 37° in the presence of 1 μM DEA/NO. To test for irreversibility of ODQ-induced inhibition, sGC was preincubated for 5 min, diluted 10-fold with the assay mixture, and incubated for ≤25 min in the presence of 1 μM DEA/NO. At the indicated time points, aliquots were removed for determination of cGMP. Data represent mean ± standard error values of three separate experiments.

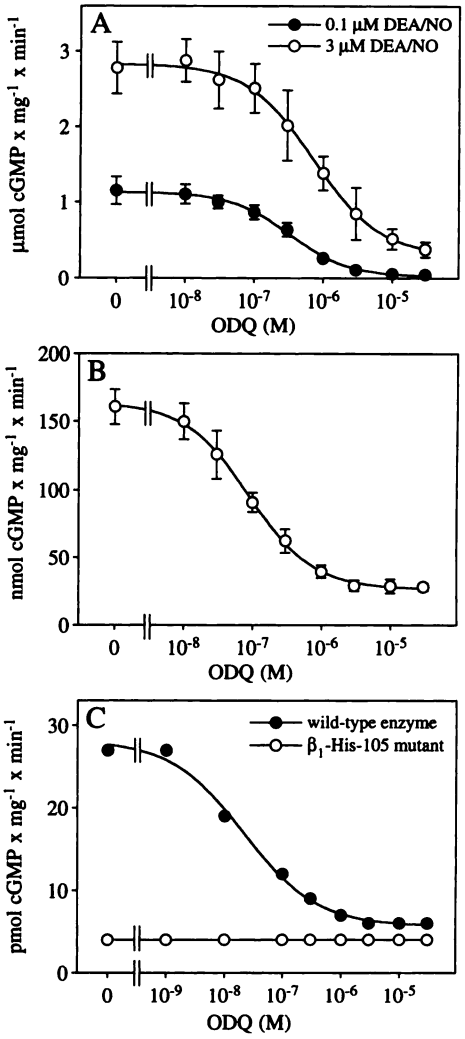

Fig. 1. ODQ inhibits both NO-stimulated and basal sGC in a concentration-dependent manner. A, Inhibition of DEA/NO-stimulated sGC. Purified sGC (45 ng) was incubated at 37° for 10 min with increasing concentrations of ODQ in the presence of 0.1 μM or 3 μM DEA/NO as described in Experimental Procedures. Data are mean ± standard error values of three experiments. B, Inhibition of basal sGC activity. Purified sGC (0.2 μg) was incubated at 37° for 20 min with increasing concentrations of ODQ as described in Experimental Procedures. Data are mean ± standard error values of three experiments. C, ODQ inhibits wild-type enzyme but not the β_1/His105 mutant of sGC. Cytosolic protein (50 μg) of COS-7 cells transfected with α_1/β_1 (●) or α_1/β_1/His105 (○) was incubated at 37° for 10 min in the presence of increasing concentrations of ODQ as described in Experimental Procedures. One of two similar experiments is shown.

Light absorbance spectroscopy. Purified sGC (3 μg in 0.1 ml) was incubated for 5 min at ambient temperature in the presence of 10 μM DEA/NO, 5 μM ferricyanide, 0.03–0.3 mM ODQ, or vehicle and injected onto a fast performance liquid chromatography set-up consisting of a 0.5 × 2 cm DEAE-FastFlow Sepharose (Pharmacia, Vienna, Austria) and a 0.5 × 10 cm Sephadex G50-fine (Sigma) column connected in series. Flow was maintained with a Pharmacia/LKB HPLC pump at 0.15 ml/min (observed back pressure, <1 p.s.i.)

in 50 mM triethanolamine/HCl buffer, pH 7.0, containing 300 mM NaCl. Spectra were recorded with a Waters photodiode-array UV monitor in a 8-μl flow cell. Sampling time was 50 msec; 10–20 separate scans were accumulated and averaged to reduce background noise.

Results

The potency of ODQ to inhibit NO-stimulated sGC was determined in the presence of two concentrations of DEA/NO. In the presence of a maximally active concentration of the NO donor (3 μM), cGMP accumulation was inhibited by ODQ with an IC_{50} of 0.72 ± 0.18 μM (Fig. 1A). A 30-fold lower concentration of DEA/NO (0.1 μM) induced ~40% of maximal cyclase stimulation. Under these conditions, the IC_{50} of ODQ was decreased ~2.5-fold to 0.28 ± 0.05 μM. The increased potency of ODQ at lower NO concentrations pointed to a competition between the inhibitor and NO. This is also suggested by results obtained at higher concentrations of the inhibitor: 1 μM ODQ, for example, produced almost complete inhibition in the presence of 0.1 μM DEA/NO but only ~50% inhibition in the presence of a 30-fold higher concentration of the NO donor.

Interestingly, sGC activity measured in the absence of added NO or NO donors was also inhibited by ODQ. As shown in Fig. 1B, basal enzyme activity was reduced by the quinoxalin with an IC_{50} of 84 ± 15 nM, but inhibition was not complete. Even at high ODQ concentrations (≥30 μM), ~20% of residual cyclase activity remained unaffected by the inhibitor. These results indicated that sGC is slightly stimulated even in the absence of added NO under normal environmental conditions. To determine whether this "autoactivation" of the enzyme was heme dependent, we recorded ODQ concentration-response curves with both wild-type bovine lung sGC and an NO-insensitive, heme-free mutant of the enzyme (β_1/His105). As shown in Fig. 1C, ODQ inhibited basal activity of the recombinant wild-type enzyme in cytosols of transfected COS-7 cells down to ~20% of control values. The remaining activity was virtually identical to the basal, ODQ-insensitive activity of the heme-free mutant, strongly suggesting that autoactivation of sGC is a heme-dependent process that is antagonized by ODQ.

In another series of experiments, sGC was stimulated with increasing concentrations of the NO donors DEA/NO and GSNO in the presence of fixed concentrations of ODQ. As shown in Fig. 2A, DEA/NO increased sGC activity to 3.49 ± 0.07 μmol of cGMP/mg/min, and half-maximal enzyme stimulation was observed with 82 ± 20 nM of the NO donor. Presence of ODQ induced a rightward shift of the concentration-response curve and a concomitant reduction in maximal cyclase activity. The EC_{50} values of DEA/NO increased from 82 ± 20 nM to 0.32 ± 0.11 μM and 1.87 ± 1.02 μM in the presence of 0.3 μM and 1 μM ODQ, respectively. Fig. 2B shows that ODQ had a similar effect on GSNO-stimulated sGC. Again, ODQ reduced both the apparent affinity of the enzyme for the NO donor and maximal specific activity.

Next, we investigated whether binding of ODQ results in inactivation of sGC. The cyclase was preincubated for ≤20 min in the absence or presence of 0.3 μM QDQ, followed by determination of DEA/NO-stimulated enzyme activity in the 10-fold-diluted aliquots. The resulting final ODQ concentrations of 30 nM had no significant effect, as revealed by the data shown in Fig. 1A. As shown in Fig. 3A, 0.3 μM ODQ induced a time-dependent inactivation of the enzyme to ~10% of control values, with a half-life of ~3 min. To test for reversibility of inactivation, sGC was preincubated with or without 0.3 μM ODQ (5 min) followed by incubation of the 10-fold diluted samples in the presence of DEA/NO for ≤25 min. As shown in Fig. 3B, the reaction rates of both control incubations and ODQ-pretreated enzyme were linear for ≥25 min. Reaction rates were decreased to ~30% of control on preincubation with the inhibitor, and sGC showed no tendency to recover from the pretreatment within the time frame of incubation.

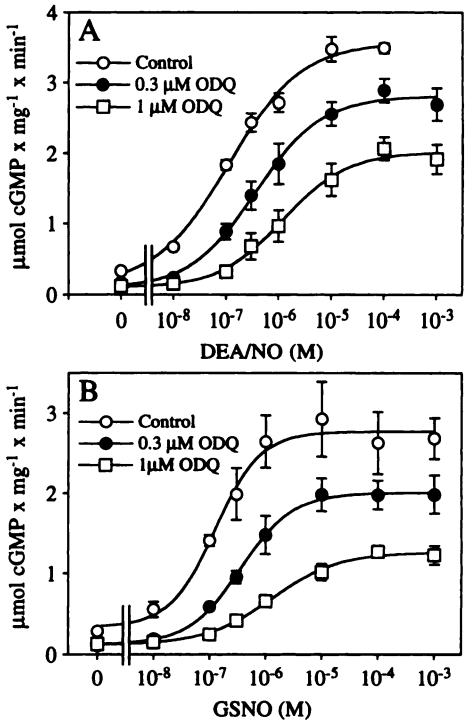

Fig. 2. ODQ antagonizes NO stimulation of sGC. A, Effect of ODQ on sGC stimulation by DEA/NO. Purified sGC (45 ng) was incubated with increasing concentrations of DEA/NO in the absence of ODQ and in the presence of 0.3 or 1 μM of the inhibitor as described in Experimental Procedures. Data are mean ± standard error values of three experiments. B, Effect of ODQ on sGC stimulation by GSNO. Purified sGC (45 ng) was incubated with increasing concentrations of GSNO in the absence of ODQ and in the presence of 0.3 or 1 μM of the inhibitor as described in Experimental Procedures. Data are mean ± standard error values of three experiments.

Based on the above observations, we speculated that ODQ may be a heme-site inhibitor of sGC, and we investigated this with the use of light absorbance spectroscopy. Fig. 4 shows the effect of DEA/NO (10 μM), ferricyanide (5 μM), and ODQ (0.3 mM) on the spectral properties of native sGC. In accordance with previous reports (7, 10), the native enzyme exhibited a sharp Soret peak at 431 nm that was shifted to 398 nm in the presence of the NO donor DEA/NO. Oxidation of the heme with ferricyanide resulted in a shift of the Soret band to 393 nm. ODQ induced a similar spectral shift, with the Soret maximum being identical to that of the ferricyanide-treated enzyme (393 nm) and an additional small shoulder at 431 nm.

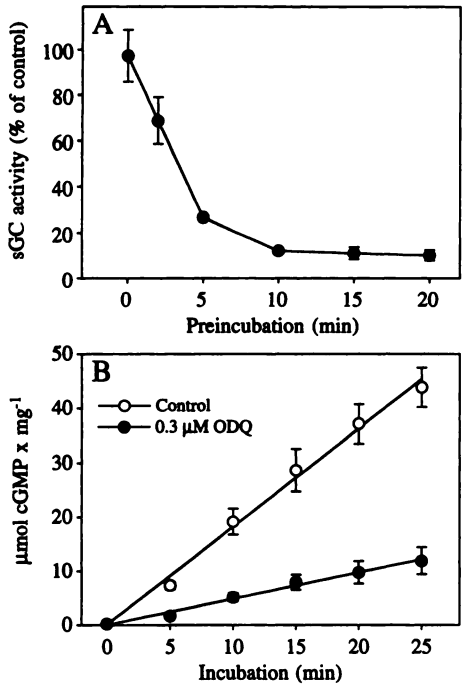

Fig. 3. ODQ produces an apparently irreversible inactivation of sGC. A, Inactivation of sGC by preincubation with ODQ. Purified sGC (0.23 μg) was preincubated at 37° in the absence or presence of 0.3 μM ODQ. At the indicated time points, aliquots were removed, diluted 10-fold in the sGC assay mixture described in Experimental Procedures, and incubated at 37° for 10 min in the presence of 1 μM DEA/NO. The results are expressed as percent activity of controls that had received vehicle instead of ODQ. Data are mean ± standard error values of three experiments. B, Inactivation of sGC by ODQ is irreversible. Purified sGC (0.23 μg) was preincubated at 37° for 5 min in the absence or presence of 0.3 μM ODQ, followed by 10-fold dilution of the sample and incubation in the presence of 1 μM DEA/NO for the indicated periods of time. Data are mean ± standard error values of three experiments.

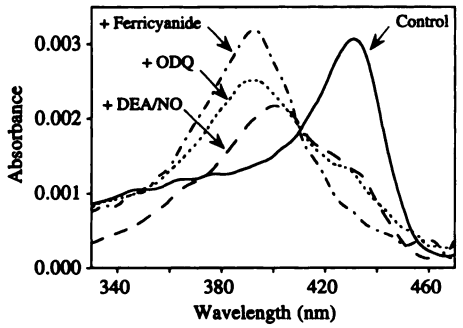

Fig. 4. Light absorbance spectroscopy of sGC pretreated with ODQ, DEA/NO, and ferricyanide. Purified sGC (3 μg) was incubated for 5 min at ambient temperature in the presence of ODQ (0.3 mM), DEA/NO (10 μM), ferricyanide (5 μM), or vehicle. Samples were subjected to sequential anion exchange and gel filtration chromatography and analyzed spectroscopically with a photodiode-array detector as described in Experimental Procedures.

Discussion

The novel and highly selective sGC inhibitor ODQ provides a powerful and unique tool for investigating the role of NO/cGMP signaling in biological systems. Phorbol ester-stimulated endothelin-1 secretion from endothelial cells, for example, was shown to be inhibited by NO donors but not by ODQ, suggesting that NO regulates this process in a cGMP-independent fashion (19). Similarly, selective inhibition by ODQ of cGMP formation in bovine pulmonary arteries revealed the existence of a cGMP-independent component of NO-mediated relaxation in this tissue (20).

In view of the broad applicability and wide impact of ODQ, we investigated the molecular mechanism accounting for sGC inhibition. Using the purified bovine lung enzyme, we obtained convincing evidence that binding of ODQ is competitive with NO and leads to an apparently irreversible inactivation of the enzyme. Although the ODQ binding site of sGC remains to be identified, the competition with NO and the spectral shift of the Soret indicate that ODQ acts as heme-site inhibitor. The similarity between the absorbance spectrum of the ODQ-treated enzyme and that of ferricyanide-treated sGC suggests that ODQ oxidizes the ferrous heme group to the ferric form. Because ferric heme is thought to have only poor affinity for NO, oxidation of ferrous heme may fully account for the inactivation of sGC by ODQ, but the spectral data suggest that the action of ODQ may be more complex. Even at a concentration of ODQ as high as 0.3 mM, the spectrum of sGC exhibited a shoulder at 431 nm. This shoulder may be due to the presence of residual amounts of native enzyme, albeit the apparently irreversible manner of inhibition renders it unlikely that the enzyme was partially reduced back to the ferrous form subsequent to removal of ODQ. Alternatively, the shoulder could be due to an unusual spectral feature of the heme. Future studies with electron paramagnetic resonance or Raman spectroscopy may shed light on the precise structure of the ODQ-modified heme. In any case, our data suggest that ODQ binds directly to the heme or to an adjacent site essentially involved in heme-dependent stimulation of sGC.

Initially, inhibition by ODQ of basal sGC activity, i.e., the enzyme activity determined in the absence of added NO or NO donors, seemed to argue against the heme as the target site of this drug. However, we consistently observed a low residual enzyme activity that was insensitive to ODQ. Most interestingly, the remaining activity was virtually identical to the activity of the NO-insensitive mutant α_1/β_1/His105 (see Fig. 1C). Thus, it is very likely that sGC is stimulated ~5-fold even in the absence of added NO. The stimulation seems to be heme dependent and is accordingly antagonized by the heme-site inhibitor ODQ.

Inactivation of purified sGC by ODQ seemed to be irreversible, even though the inhibitory effect of the quinoxalin was clearly reversible in brain slices (18) and endothelial cells (20). This discrepancy suggests that cells may be able to reactivate sGC. If existing, such a cellular mechanism of sGC reactivation could play a crucial role in the physiological activation/deactivation cycle of the enzyme and certainly warrants further investigation.

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References

1. Moncada, S., R. M. J. Palmer, and E. A. Higgs. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**:109–142 (1991).
2. Garthwaite, J. Neural nitric oxide signalling. *Trends Neurosci.* **18**:51–52 (1995).

3. Waldman, S., and F. Murad. Cyclic GMP synthesis and function. *Pharmacol. Rev.* **39**:163–195 (1987).
4. Ignarro, L. J. Heme-dependent activation of guanylate cyclase by nitric oxide: a novel signal transduction mechanism. *Blood Vessels* **28**:67–73 (1991).
5. Koesling, D., E. Böhme, and G. Schultz. Guanylyl cyclases, a growing family of signal transducing enzymes. *FASEB J.* **5**:2785–2791 (1991).
6. Schulz, S., P. S. T. Yuen, and D. L. Garbers. The expanding family of guanylyl cyclases. *Trends Pharmacol. Sci.* **12**:116–120 (1991).
7. Gerzer, R., E. Böhme, F. Hofmann, and G. Schultz. Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS Lett.* **132**:71–74 (1981).
8. Ignarro, L. J., J. N. Degnan, W. H. Baricos, P. J. Kadowitz, and M. S. Wolin. Activation of purified guanylate cyclase by nitric oxide requires heme: comparison of heme-deficient, heme-reconstituted and heme-containing forms of soluble enzyme from bovine lung. *Biochim. Biophys. Acta* **718**:49–59 (1982).
9. Wedel, B., P. Humbert, C. Harteneck, J. Foerster, J. Malkewitz, E. Böhme, G. Schultz, and D. Koesling. Mutation of His-105 of the β_1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. USA* **91**:2592–2596 (1994).
10. Stone, J. R., and M. A. Marletta. Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* **33**:5636–5640 (1994).
11. Stone, J. R., R. H. Sands, W. R. Dunham, and M. A. Marletta. Electron paramagnetic resonance spectral evidence for the formation of a pentacoordinate nitrosyl-heme complex on soluble guanylate cyclase. *Biochem. Biophys. Res. Commun.* **207**:572–577 (1995).
12. Yu, A. E., S. Z. Hu, T. G. Spiro, and J. N. Burstyn. Resonance raman spectroscopy of soluble guanylyl cyclase reveals displacement of distal and proximal heme ligands by NO. *J. Am. Chem. Soc.* **116**:4117–4118 (1994).
13. Burstyn, J. N., A. E. Yu, E. A. Dierks, B. K. Hawkins, and J. H. Dawson. Studies of the heme coordination and ligand binding properties of soluble guanylyl cyclase (sGC): characterization of Fe(II)sGC and Fe(II)sGC(CO) by electronic absorption and magnetic circular dichroism spectroscopies and failure of CO to activate the enzyme. *Biochemistry* **34**:5896–5903 (1995).
14. Traylor, T. G., and V. S. Sharma. Why NO? *Biochemistry* **31**:2847–2849 (1992).
15. Stone, J. R., and M. A. Marletta. Heme stoichiometry of heterodimeric soluble guanylate cyclase. *Biochemistry* **34**:14668–14674 (1995).
16. Mayer, B., F. Brunner, and K. Schmidt. Inhibition of nitric oxide synthesis by methylene blue. *Biochem. Pharmacol.* **45**:367–374 (1993).
17. Luo, D., S. Das, and S. R. Vincent. Effects of methylene blue and LY83583 on neuronal nitric oxide synthase and NADPH-diaphorase. *Eur. J. Pharmacol.* **290**:247–251 (1995).
18. Garthwaite, J., E. Southam, C. L. Boulton, E. B. Nielsen, K. Schmidt, and B. Mayer. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one. *Mol. Pharmacol.* **48**:184–188 (1995).
19. Brunner, F., H. Stessel, and W. R. Kukovetz. Novel guanylyl cyclase inhibitor, ODQ reveals role of nitric oxide, but not of cyclic GMP in endothelin-1 secretion. *FEBS Lett.* **376**:262–266 (1995).
20. Brunner, F., K. Schmidt, E. B. Nielsen, and B. Mayer. Novel guanylyl cyclase inhibitor potently inhibits cyclic GMP accumulation in endothelial cells and relaxation of bovine pulmonary artery. *J. Pharmacol. Exp. Ther.* **277**:48–53 (1996).
21. Humbert, P., F. Niroomand, G. Fischer, B. Mayer, D. Koesling, K.-D. Hinsch, G. Schultz, and E. Böhme. Preparation of soluble guanylyl cyclase from bovine lung by immunoaffinity chromatography. *Methods Enzymol.* **195**:384–391 (1991).
22. Schultz, G., and E. Böhme. Guanylate cyclase. GTP pyrophosphate-lyase (cyclizing), E.C. 4.6.1.2., in *Methods of Enzymatic Analysis* (H. U. Bergmeyer, J. Bergmeyer, and M. Graßl, eds.). Verlag Chemie, Weinheim, Germany, 379–389 (1984).

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