Selective Guanylyl Cyclase Inhibitor Reverses Nitric Oxide–Induced Vasorelaxation

Linda J. Olson, Edward T Knych Jr, Thomas C Herzig, James G Drewett

Abstract  Effects of a novel soluble guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-c]quinazolin-1-one (ODQ), were characterized on guanylyl cyclase activity in cytosolic fraction of COS-7 cells overexpressing the α1 and β1 subunits of the rat soluble enzyme. ODQ was a noncompetitive inhibitor of soluble guanylyl cyclase with respect to Mn2+ or Mn2+-GTP and was a mixed competitive/noncompetitive inhibitor with respect to nitric oxide (NO) donation. ODQ (10 μmol/L) reduced data nonoate-stimulated cGMP production in COS-7 cells overexpressing soluble guanylyl cyclase and in rat aortic vascular smooth muscle cells. ODQ did not inhibit particulate forms of the enzyme rat guanylyl cyclase-A, -B, or -C, did not block NO synthase, and did not auto-oxidize data nonoate-donated NO in the presence of cells at physiological pH. Therefore, ODQ is a selective inhibitor of soluble guanylyl cyclase. Using ODQ in isolated aortic ring preparations, we tested the hypothesis that soluble guanylyl cyclase mediates vasorelaxant activity associated with NO Phenylephrine (100 nmol/L)-precontracted, isolated rat aortas were relaxed in a concentration-dependent manner by data nonoate (0.01 to 100 μmol/L) and nitroglycerin (0.01 to 300 μmol/L). ODQ (10 μmol/L) attenuated data nonoate- and nitroglycerin-mediated relaxation of contracted aortas. ODQ had no effect on nitrate/nitrite–depleted aortic relaxation. These results support the hypothesis that soluble guanylyl cyclase mediates vasorelaxant activity associated with nitric oxide (Hypertension. 1997;29 [part 2]:254-261.)

Key Words  cyclic GMP • endothelium-derived relaxing factor • guanylyl cyclase • nitric oxide • nitric oxide synthase • nitrate/nitrite peptides • vasodilatation

Nitric oxide is now known to be the major endothelium-derived relaxing factor. Several vasodilators, including bradykinin, acetylcholine, and serotonin, are thought to have activity by virtue of their effects to stimulate endothelial cell NOS. Several studies over the years have suggested that the vasodilatory actions of NO are mediated by increases in cGMP and NO and several precursors of the radical activated soluble forms of guanylyl cyclase, and membrane permeable analogs of cGMP vasodilated blood vessels. In addition, in most studies NO-stimulated production of cGMP correlates with the NO-mediated vasodilation. However, NO-induced smooth muscle relaxation has been dissociated from increases in cGMP in some studies. Potential explanations for the latter observation are that NO also stimulates guanylyl cyclase activity in cell types other than vascular smooth muscle (ie, adventitia, fibroblasts, autonomic nerves, and/or endothelium), that current assays for vascular contractile responses are significantly more sensitive than the measurement of cGMP concentrations within cellular compartments associated with vasoactivity, and that NO may mediate vascular effects independent of guanylyl cyclase stimulation.

Several groups have used either methylene blue or LY83583 as sGC inhibitors to focus on the latter possibility by attempting to dissociate NO-mediated smooth muscle relaxation from increases in enzyme activation. Both of these compounds appear to block the vascular relaxation and lower cGMP concentrations associated with vasoactive agents that stimulate NOS or NO donors, including nitroglycerin, sodium nitroprusside, and 3-morpholino-sydnonimine (ie, SIN-1). Therefore, methylene blue and LY83583 are not truly selective sGC inhibitors, and their effects on the NO/sGC system are likely completely or partially explained by indirect actions. Methylene blue is now known not only to inhibit NOS but also to lower NO concentrations by the production of superoxide anion. LY83583 was first reported to reduce cGMP concentrations in the guinea pig lung by an unknown mechanism not involving a reduction of SGC activity. Regardless, several studies over the last decade have used it to form conclusions about the relationship of NO- and sGC-associated cellular events, including vasorelaxation in aortic rings. An early report demonstrated that LY83583 blocked the production of endothelium-derived relaxing factor, and a very recent study has demonstrated that, like methylene blue, LY83583 also can act as an inhibitor of NOS in rat cerebellar homogenates. The latter study, as well as others, has revealed more parallels between methylene blue and LY83583, including the production of superoxide anion, to explain the effects of the latter compound to decrease NOS activity or auto-oxidize NO. Therefore, it is impossible to use LY83583 and/or methylene blue to differentiate between direct inhibition of SGC activity (ie, effects on the receptor) or reductions in NOS or NO concentrations (ie, effects on ligand concentrations).

Garthwaite et al recently reported the identification of a potent, selective inhibitor of NO-sensitive guanylyl cyclase called ODQ. They demonstrated that ODQ inhibited purified NO-stimulated SGC activity in a noncompetitive manner with respect to the substrate GTP and...
Selected Abbreviations and Acronyms

8-bromo-cGMP = 8-bromo-guanosine 3' 5'-monophosphate
ANP = type A natriuretic peptide
BTP = type B natriuretic peptide
CNP = type C natriuretic peptide
GC-A, -B, -C = guanylyl cyclase-A, -B, -C
LY83583 = 6-amino-5,8-quinolinedione
NO(S) = nitric oxide (synhase)
ODQ = 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-
one
sGC = soluble guanylyl cyclase

Further determined that ODQ did not deactivate reactive NO, did not produce superoxide anions, and did not block the sGC-independent effect of NO to induce macrophage cytotoxicity. 18 ODQ blocked NO-donor stimulation of sGC in intact endothelial cells and had no effect on ANP stimulation of particulate guanylyl cyclase as assessed by measurement of cGMP production by radiomunnoasay. 19 ODQ also did not inhibit forskolin-stimulated adenyl cyclase cyclase in endothelial cells. 18 Taken together, these initial results suggested that ODQ was potentially a much better agent for assessing the involvement of sGC in NO effects than either methylene blue or LY83583.

In the present study, we characterized the effects of ODQ on Mn2+, Mn2+-GTP–, and data nonoate-stimulated sGCs in the cytoplasmic fraction from COS-7 cells overexpressing the α1 and β1 subunits of the rat enzyme (COS-7 sGC cells) Furthermore, we also examined the effect of ODQ on cells overexpressing particulate forms of the enzyme GC-A, GC-B, and GC-C, which act as receptors for ANP, CNP, and heat-stable enterotoxin, respectively. Following this characterization, we used ODQ in rat isolated aortic rings to test the hypothesis that sGC mediates vasorelaxant activity associated with NO donation.

Methods

General

Unless specified in the following text, common laboratory reagents used in this study were purchased from either Sigma Chemical Co or Fisher Scientific. All protease inhibitors were from Sigma Chemical Co as well unless otherwise noted.

Cell Culture and Transfection

Cells were maintained in a humidified 95% air–5% CO2 water-jacketed incubator (Nuaire) at 37°C. HEK-293 cells overexpressing stable transfectants of rat GC-A, GC-B, and GC-C (HEK-293 GC-A, -B, and -C cells, respectively) were kindly provided by Dr David L Garbers (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center [Dallas]) and cultured as described. 20 22 COS-7 cells were cultured as previously described23 and transfected with the α1 and β1 subunits of sGC by the DEAE/dextran method. 24 The rat α1 and β1 subunit cDNAs in the mammalian expression vector (pCMV2) were also provided by Dr D.L. Garbers. 25 The expression of active sGC requires the cotransfection of both α and β subunits. 25-29 Cells were cotransfected with 5 μg of each subunit. Mock-transfected cells were transfected with 10 μg pCMV2. Cells were allowed to grow for 48 hours before experimentation. Expression of both subunits was confirmed by beta nonoate (nonoate) (Cayman Chemical)–stimulated production of cGMP in whole cells or guanylyl cyclase activity in soluble fractions from these cells.

Rat Aortic Vascular Smooth Muscle Cells

Cells were isolated according to the method of Digho et al. 30 Male Sprague-Dawley rats (300 to 400 g) were given a lethal intraperitoneal injection of sodium pentobarbital (50 mg/kg), and a thoracotomy was performed to remove the full-length thoracic aorta. These manipulations were performed in accordance with the Medical College of Wisconsin Animal Care Committee guidelines. Ring explants (1 to 1.5 mm thick) were placed into 24-well tissue culture plates (Falcon) containing Dulbecco’s modified Eagle’s medium (Gibco Life Technologies) supplemented with 10% defined fetal bovine serum (Hyclone Lab) Explants were removed 3 to 4 days later, and the cells remaining on the dash were grown to confluence. Growth medium was the same as that above during explantation with the following supplements: 2 mmol/L L-glutamine, 100 U/mL penicillin, and 70 μg/mL streptomycin (Gibco). The smooth muscle identity of these cultures was verified morphologically (ie, hill and valley growth pattern) and by immunohistochemical staining for both the expression of smooth muscle–specific α-actin and the absence of factor VIII antigen. Immunohistochemical staining verified that the cells were more than 97% smooth muscle. The cells were used in passages 4 to 6.

Bovine Coronary Artery Endothelial Cells

Endothelial cells were kindly provided by Dr William B Campbell (Medical College of Wisconsin [Milwaukee]). The isolation and culturing conditions of these cells have been previously described31 and were followed throughout these studies.

Whole-Cell cGMP Production Studies

Experiments testing ODQ effects (Tocris Cookson) on particulate guanylyl cyclases were examined in HEK-293 GC-A, -B, and -C cells overexpressing each of these enzymes. Those testing the effects of ODQ on sGC were performed in both COS-7 sGC cells and rat aortic vascular smooth muscle cell primary cultures. All experiments were performed in two paired 24-well plates (Falcon or Sarstedt). One plate received ODQ treatment and the other ODQ vehicle (ethanol). Before initiation of experiments, confluent plates of cells were rinsed twice with 5 mL PBS (pH 7.4, 37°C). Cells were preincubated at 37°C for 10 minutes with 25 mmol/L isobutylmethylxanthine and ODQ vehicle or 10 μmol/L ODQ in HEPES (25 mmol/L)-buffered (pH 7.4) Dulbecco’s modified Eagle’s medium (Medical College of Wisconsin Tissue Culture Facility). Preincubation medium was removed and replaced with fresh buffer of the same components with the appropriate guanylyl cyclase agonist for 5 minutes at 37°C. Four paired experiments were performed per set of ODQ vehicle- and ODQ-treated plates. HEK-293 GC-A, -B, and -C cells were treated with vehicle (water) and increasing concentrations of ANP, CNP, and heat-stable enterotoxin, respectively. COS-7 sGC cells and rat aortic vascular smooth muscle cells were treated with nonoate vehicle (0.01 mol/L NaOH) and increasing concentrations of the NO donor or its by-product, ethylene oxide (Aldrich Chemical Co). Specifically, vehicle or one of five concentrations of agonist was given per well (ANP, CNP, heat-stable enterotoxin vehicle, 10–10, 10–9, 10–8, 10–7, or 10–6 mol/L, nonoate/ethylene oxide vehicle, 10–5, 10–4, 10–3, 10–2, or 10–1 mol/L) After the 5-minute incubation period, the supernatants were aspirated, and the guanylyl cyclase reaction was terminated by the addition of 0.5 mL of 1 mol/L perchloric acid. Antiserum for cGMP was graciously supplied by Dr D.L. Garbers. 32 Samples containing cGMP were subjected to alumina–Dowex ion-exchange column purification32 and radiomunnoassay as previously described. 33 34 Resultant samples were counted on a Packard Cobra II Auto- Gamma Counter.

Guanylyl Cyclase Assay

COS-7 sGC Cells

Two days after transfection, COS-7 sGC cells were harvested and resuspended in 10 mmol/L HEPES (pH 7.4), 150 mmol/L.
NaCl, 1 mmol/L EDTA, 1.5 μmol/L aprotinin, 10 μmol/L E-64 (Boehringer Mannheim Biochemicals), and 0.1 μmol/L PMSF. After homogenization, cytosolic proteins were isolated by centrifugation (15 000g, 30 minutes, 4°C) Guanylyl cyclase activity was determined in 100-μL reactions containing 5 μg soluble protein, 40 mmol/L trisethanolamine HCl, pH 7.5, 0.25 mmol/L isobutylmethylxanthine, 2 mmol/L DTT, 1 μmol/L PMSF, and 1 μmol/L peptatin A. In kinetic studies, 5 minute reactions were performed in the presence or absence of ODQ (10 μmol/L) at 37°C, containing the varied or fixed concentrations of Mn^2+, Mn^2+-GTP, and/or nonoate as described in "Results" and in Table 1. The synthesis of cGMP was verified to be linear over the 5-minute course of the experiment. Reactions were terminated, and cGMP was purified and quantified as described above.

For assays quantifying the conversion of radiolabeled GTP to cGMP, the reaction mixture also contained 1 μCi [α-32P]GTP (ICN Radiochemicals) and either 600 nmol/L Mn^2+ per 100 μmol/L GTP or 1.1 mmol/L Mn^2+ per 1 mmol/L GTP. Proteins were preincubated (10 minutes, 37°C) either in the presence of 10 μmol/L ODQ or vehicle (ethanol) before the addition of Mn^2+-GTP. Reactions were terminated after 5 minutes by the addition of 0.5 mL 110 mmol/L zinc acetate and 0.5 mL 110 mmol/L sodium bicarbonate. Samples were centrifuged (1000g, 5 minutes), and supernatants were purified over alumina columns. Eluates containing [32P]cGMP were subjected to scintillation counting on Packard Instrument Tri-Carb 2100TR.

**Rat Aortas**

Male Sprague-Dawley rats (300 to 400 g) were given a lethal intraperitoneal injection of sodium pentobarbital (50 mg/kg). These manipulations were performed in accordance with the Medical College of Wisconsin Animal Care guidelines. Thoracic aortas were removed and rings prepared in 5-mL tissue baths as previously described. Contractile responses were monitored using Grass instrumentation as described.

After the precontraction and pretreatments, one ring of each pair received ODQ vehicle (ethanol) and the other ring received ODQ (10 μmol/L) 15 minutes before the addition of the α-selective adenoreceptor agonist phenylephrine (100 μmol/L). The rings were allowed to maximally contract over the next 15 minutes before the addition of vehicle and increasing cumulative concentrations of nonoate (10^(-8) to 3 × 10^(-5) mol/L), ANP (10^(-10) to 10^(-6) mol/L), CNP (10^(-10) to 10^(-9) mol/L), nitroglycerin (10^(-8) to 3 × 10^(-5) mol/L), isoprotrenol (10^(-8) to 10^(-5) mol/L), bimatapril (10^(-10) to 10^(-6) mol/L), or L-arginine (10^(-3) to 10^(-4) mol/L) every 4 minutes or until a plateau was reached following each addition of relaxant agonist. Because nonoate must be stored in 0.01 mol/L NaOH to avoid hydrolysis, appropriate dilutions of the NO donor in Krebs' buffer were made approximately 1 minute before addition to the tissue baths to eliminate the potential for alkalization of the bathing medium and associated nonspecific effects.

**NOS Activity**

Samples containing 0.1 mg lysate protein from transiently transfected COS-7 G6C cells or endothelial cells were preincubated (10 minutes, 37°C) in the presence of 10 μmol/L ODQ, 1 mmol/L N^\text{V}-nitro-L-arginine, 0.1 mmol/L methylene blue, or respective vehicle treatment NOS activity was measured by monitoring the conversion of L-[3H]-arginine to L-[3H]-citrulline as described above. Reactions were terminated, and L-[3H]-citrulline were determined by scintillation counting (Packard Tri-Carb 2100TR). Data were expressed as femtomoles of L-[3H]-citrulline formed per milligram of lysate protein per minute.

**Vasorelaxation Studies**

Male Sprague-Dawley rats (300 to 400 g) received a lethal intraperitoneal injection of sodium pentobarbital (50 mg/kg). These manipulations were performed in accordance with the Medical College of Wisconsin Animal Care guidelines. Thoracic aortas were removed and rings prepared in 5-mL tissue baths as previously described. Contractile responses were monitored using Grass instrumentation as described.

After the precontraction and pretreatments, one ring of each pair received ODQ vehicle (ethanol) and the other ring received ODQ (10 μmol/L) 15 minutes before the addition of the α-selective adenoreceptor agonist phenylephrine (100 μmol/L). The rings were allowed to maximally contract over the next 15 minutes before the addition of vehicle and increasing cumulative concentrations of nonoate (10^(-8) to 3 × 10^(-5) mol/L), ANP (10^(-10) to 10^(-6) mol/L), CNP (10^(-10) to 10^(-9) mol/L), nitroglycerin (10^(-8) to 3 × 10^(-5) mol/L), isoprotrenol (10^(-8) to 10^(-5) mol/L), bimatapril (10^(-10) to 10^(-6) mol/L), or L-arginine (10^(-3) to 10^(-4) mol/L) every 4 minutes or until a plateau was reached following each addition of relaxant agonist. Because nonoate must be stored in 0.01 mol/L NaOH to avoid hydrolysis, appropriate dilutions of the NO donor in Krebs' buffer were made approximately 1 minute before addition to the tissue baths to eliminate the potential for alkalization of the bathing medium and associated nonspecific effects. Nitroglycerin was manufactured by Parke-Davis, and bimatapril was manufactured by Merck KGaA Co and graciously provided by Dr Garrett J Gross (Medical College of Wisconsin [Milwaukee]).

In the presence of paired ODQ vehicle- and ODQ-treated aortic rings, we also tested not only for potential desensitization to phenylephrine-induced contractions over time but also for possible time-dependent nonoate-vehicle effects on contractile activity. Two sets of paired rings were contracted with phenylephrine or without nonoate vehicle treatment (i.e., appropriate dilutions of the nonoate storage solution, 0.01 mol/L NaOH, amin to those above for nonoate treatment) over a 65-minute period every 4 to 8 minutes to approximate the time necessary for complete responsiveness to each nonoate addition during typical experiments Nonoate (1 mmol/L) was added to the paired vessels after the completion of these time-dependent experiments to verify the capacity of each ring to respond to the NO donor.

**Auto-oxidation of NO**

NO measurements were performed using a commercially available Clark-type electrode (ISO-NO, World Precision Instruments) and documented on a strip-chart recorder. The electrode was calibrated daily using acidified potassium nitrite as the NO donor according to the manufacturer's instructions. All measurements were made under constant stirring. The effects of ODQ or the superoxide generating system xanthine/xanthine oxidase were studied using a small plastic vial containing 2 mL Krebs' bicar-

| Table 1. Effect of ODQ on Kinetic Parameters of Guanylyl Cyclase Activity in the Cytosolic Fraction From COS-7 Cells Transiently Transfected With α, and β, Subunits of Rat Soluble Enzyme |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **ODQ Vehicle**                 | **Fixed**                       | **Varied**                      | **ODQ Vehicle**                 | **Fixed**                       | **Varied**                      |
| Mn^2+                           | Mn^2+-GTP                       | Mn^2+-4GTP                      | Mn^2+/Mn^2+-GTP                 | Nono                            |
| Vmax (nmol cGMP formed/min/mg protein) | Km (μmol/L) | Vmax (nmol cGMP formed/min/mg protein) | Km (μmol/L) | Vmax (nmol cGMP formed/min/mg protein) | Km (μmol/L) |
| 5.0±0.20                       | 0.60±0.12                      | 5.0±0.20                       | 0.60±0.12                       | 5.0±0.20                       | 0.60±0.12                      |
| 8.6±0.20                       | 0.61±0.08                      | 7.0±0.10                       | 0.23±0.02                       | 8.1±0.20                       | 0.32±0.04                      |
| 8.1±0.20                       | 0.32±0.04                      | 185.58                         | 0.20±0.06                       | 7.0±0.10                       | 0.23±0.02                       |

Vmax (nmol cGMP formed/min/mg protein) and Km (μmol/L) were calculated by double-reciprocal plots. Values are means±SEM; n, number of independent assays; Mn^2+ 125 μmol/L, Mn^2+-GTP 2 to 50 μmol/L, Mn^2+-4GTP 2 mmol/L, Mn^2+/Mn^2+-GTP 125/12.5 μmol/L, nonoate 0.05 to 1 mmol/L. Paired t test comparison of ODQ vehicle (ethanol) vs test 0.05.
Km and Vmax values from the kinetic assays of enzyme activity from the cytoplasmic fraction of COS-7 sGC cells were determined by traditional double-reciprocal conversions. Statistical comparisons between ODQ-treated and ODQ vehicle-treated preparations for both Km and Vmax values were made using Student’s paired t test. In all figures and tables, asterisks denote the statistical significance (**P<.05, ***P<.01, ****P<.001) of a specific treatment when compared with a matched control group (ie, vehicle treatment).

Results

Effect of ODQ on NO-Donor-Mediated Stimulation of cGMP Production

These experiments were designed to compare the effects of nonoate-mediated cGMP production in both rat aortic vascular smooth muscle cells (Fig 1A) and COS-7 sGC cells (Fig 1B). In both rat aortic vascular smooth muscle cells (Fig 1A) and COS-7 sGC cells (Fig 1B), nonoate (10⁻³ to 10⁻¹ mol/L) resulted in a concentration-dependent (ANOVA P<.0001) increase in cGMP production with an EC₅₀ value of approximately 30 μmol/L. In aortic cells (Fig 1A), no greater effect was obtained after 10 mmol/L nonoate treatment (n=2, L.J.O., T.C.H., J.G.D., unpublished data, 1996), similar to the results in COS-7 sGC cells (Fig 1B). Therefore, in both cell types, 1 mmol/L nonoate resulted in a maximal effect on cGMP production We chose to use a 10⁻µmol/L concentration of ODQ in these studies on the basis of previous results demonstrating that concentration to be maximally effective. Treatment with ODQ reduced the ability of nonoate to increase cGMP levels in COS-7 cells (Fig 1B) and completely blocked the nonoate effect in the vascular smooth muscle cells (Fig 1A) (both by ANOVA, P<.0001). We also found that ODQ completely prevented maximal nonoate (1 mmol/L) stimulation of guanylyl cyclase activity in soluble fractions from rat thoracic aorta, consistent with the results in aortic smooth muscle cell cultures (L.J.O., J.G.D., unpublished data, 1996).

Nonaotae is known to hydrolyze into equal molar amounts of NO and ethylamine at neutral pH. As a control for a by-product effect, ethylamine (10⁻³ to 10⁻¹ mol/L) was tested for any effect on sGC activity. It was found to be without effect (L.J.O., J.G.D., unpublished data, 1996).

Effect of ODQ on Nonaote- and Nitroglycerin-Mediated Relaxation of Phenylephrine-Preconracted Rat Aortas

These experiments were performed to determine whether ODQ blocked the vasorelaxant activity of two NO donors, nonoate and nitroglycerin. The summary data from several experiments assessing the effect of ODQ on nonoate-induced relaxation are shown in Fig 2. In the absence of ODQ pretreatment, nonoate (10⁻¹ to 10⁻⁴ mol/L) resulted in a concentration-dependent relaxation of phenylephrurine-precontracted rat aorta (IC₅₀=1 μmol/L, ANOVA, P<.0001). Complete relaxation was observed on reaching a final nonoate concentration of 10⁻⁴ mol/L in the absence of ODQ. After ODQ (10 µmol/L) treatment, the IC₅₀ for nonoate (ANOVA, P<.0001) was increased by 30-fold, and the maximal effect of nonoate was attained at 10⁻¹ mol/L and reduced by about 75%. No further relaxation...
was observed at $3 \times 10^{-3}$ mol/L nonoate (n=3). These results are consistent with a mixed competitive/noncompetitive antagonism of NO effects in the rat aorta. The same ODQ concentration also blocked the ability of nitroglycerin to relax phenylephrine-precontracted aorta in a similar manner (n=3, unpublished data, 1996). Neither the nonoate by-product, 19 ethylamine ($10^{-7}$ to $10^{-3}$ mol/L), nor the nonoate vehicle (ethanol) had any effect on basal- or phenylephrine-induced rat aortic contractions (L J O., J G D., unpublished data, 1996; Fig 2).

The nonoate-mediated relaxation in the ODQ-treated vessel also was not simply due to time-dependent loss of contractile activity, nonoate-vehicle effect, or desensitization to phenylephrine. Control experiments were performed whereby phenylephrine was added to paired ODQ-treated and ODQ vehicle–treated aortic rings. The associated phenylephrine–induced contractions were maintained with or without nonoate-vehicle treatment for the approximate 65-minute duration of the experiments in these studies (n=3). In both variations, addition of 1 mmol/L nonoate at the end of this time period resulted in complete relaxation of the ODQ vehicle–treated ring and partial relaxation (≈20% to 25%) of the ODQ-treated vessel (n=3), akin to the results of Fig 2

Effect of ODQ on sGC Activity in the Cytosolic Fraction From COS-7 sGC Cells

It has been previously shown that maximal rat lung sGC activation is obtained in the presence of excess Mn$^{2+}$ over GTP. For kinetic analysis of an enzyme system requiring an excess concentration of cation over nucleotide, the system can be viewed as having two substrates, a metal-nucleotide chelate, Mn$^{2+}$-GTP, and the metal ion, Mn$^{2+}$. Chrisman et al. have previously demonstrated that sGC is such an enzyme. Similar methods to those of the latter study were used in the following experiments assessing enzyme activation in the cytoplasmic fraction from COS-7 sGC cells.

These studies were performed to determine whether ODQ was a competitive and/or noncompetitive inhibitor of sGC with respect to NO, Mn$^{2+}$, and Mn$^{2+}$-GTP. $K_m$ and $V_{max}$ values of sGC for Mn$^{2+}$ and Mn$^{2+}$-GTP were determined at near-saturating concentrations of Mn$^{2+}$-GTP (2 mmol/L) and Mn$^{2+}$ (125 mmol/L), respectively, in the presence and absence of ODQ (Table 1). In both variations, ODQ had a statistically significant effect to lower $V_{max}$ approximately threefold with no effect on $K_m$ (Table 1). The NOS inhibitor, N$^\text{N}$-nitro-L-arginine (0.1 mmol/L) did not alter these kinetic reactions (L J O., J G D., unpublished data, 1996). These results indicate a noncompetitive inhibition with respect to both metal-nucleotide chelate or Mn$^{2+}$ independent of the presence of NO. $K_m$ and $V_{max}$ values of sGC for NO were determined in the presence and absence of ODQ with fixed concentrations of Mn$^{2+}$ (125 mmol/L) and GTP (12 5 mmol/L). As shown in Table 1, ODQ significantly lowered the $V_{max}$ and increased the $K_m$ (approximately twofold) of the enzyme for nonoate-donated NO. The latter result argues that ODQ is a mixed competitive/noncompetitive inhibitor with respect to NO donation. In all three variations, the synthesis of cGMP was linear over the 5-minute course of the experiment. Subsequent experiments also demonstrated that NOS activity was not detected in COS-7 sGC cells (see below, Table 2).

### Table 2. Effects of ODQ, N$^\text{N}$-Nitro-L-Arginine, and Methylene Blue on NOS Activity in Whole-Cell Lysates From Bovine Coronary Artery Endothelial Cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>NOS Activity, fmol citrulline formed/mg lysate protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODQ (10 µmol/L)</td>
<td>247±14</td>
</tr>
<tr>
<td>N$^\text{N}$-nitro-L-arginine (0.1 mmol/L)</td>
<td>222±10</td>
</tr>
<tr>
<td>Methylene blue (0.1 mmol/L)</td>
<td>248±5</td>
</tr>
</tbody>
</table>

Values are mean±SE for n=4. (-) indicates inhibitor vehicle treatment, (+), inhibitor treatment, and ND, not detectable.

### Lack of ODQ Effect on NOS Activity in Endothelial Cells and Absence of NOS Activity in COS-7 sGC Cells

The purpose of these experiments was to test for an effect of ODQ on NOS activity in endothelial cells and in COS-7 sGC cells. Table 2 shows that N$^\text{N}$-nitro-L-arginine and methylene blue (0.1 mmol/L each) completely block the conversion of [3H]-L-arginine to [3H]-L-citrulline in bovine coronary artery endothelial cells. ODQ (10 µmol/L), however, does not effect the same reaction, demonstrating that it does not modulate NO generation. NOS activity was not detectable in COS-7 sGC cells (Table 2).

### Effect of Xanthine/Xanthine Oxidase and ODQ on the Auto-oxidation of Nonoate-Donated NO

These studies determined whether ODQ could oxidize NO in the presence of COS-7 cells or rat aortic rings at physiological pH. In both of these preparations, the effects of the superoxide generating system xanthine/xanthine oxidase and ODQ were examined. Following treatment with 50 µmol/L nonoate in the presence of xanthine/xanthine oxidase vehicle, the concentration of NO generated was 168±15 nmol/mL (n=3) or 266±47 nmol/mL (n=2) in the presence of COS-7 cells or aorta, respectively. Combined treatment with xanthine (10 µmol/L) and xanthine oxidase (5 U/mL) lowered the levels of nonoate-donated NO from those obtained in the presence of nonoate and xanthine/xanthine oxidase vehicle (above) to 49±4.9% for COS-7 cells (n=3) and 22±3.2% (n=2) for aortic rings. In contrast, ODQ (10 µmol/L) did not result in reductions of NO concentrations, which were 105±1.3% (n=3) and 107±0.2% (n=2) of the control levels reached in the presence of both 50 µmol/L nonoate and xanthine/xanthine oxidase vehicle (above) for COS-7 cells and rat aortic rings, respectively. Xanthine (10 µmol/L) or xanthine oxidase (5 U/mL) alone had no effect on the amount of nonoate-donated NO in the presence of COS-7 cells or aortic rings (L J O., E T K, J G D., unpublished data, 1996).

### Lack of ANP-, CNP-, and Heat-Stable Enterotoxin–Stimulated cGMP Production in HEK-293 Cells Expressing Stable Transfectants of GC-A, -B, and -C, Respectively

These experiments were designed to test whether ODQ had any effect on the membrane-associated guanylyl cyclases GC-A, -B, or -C. ANP, CNP, and heat-stable enterotoxin (10$^{-10}$ to 10$^{-6}$ mol/L) result in concentration-dependent (ANOVA, P<.0001) stimulation of cGMP production in HEK-293 GC-A (Fig 3A), -B (Fig 3B), and -C (Fig 3C) cells, respectively. ODQ pretreatment had no
effect by ANOVA on the ability of ANP, CNP, or heat-stable enterotoxin to stimulate the membrane-associated guanylyl cyclases GC-A, -B, or -C (Fig 3 through C, respectively), which act as the receptors for these peptides.

Lack of ODQ Effect on Natriuretic Peptide–Induced Relaxation of Phenylephrine-Precontracted Rat Aorta

These studies tested the ability of ODQ to block the effect of two known vasorelaxant peptides, ANP and CNP, which act as agonists of the membrane-associated guanylyl cyclases GC-A and GC-B. Both ANP (10^{-9} to 10^{-7} mol/L, IC_{50}=3 nmol/L; Fig 4) and CNP (10^{-8} to 10^{-6} mol/L, IC_{50}=30 nmol/L; Fig 4) resulted in concentration-dependent vasorelaxation of precontracted rat aorta (ANOVA, P<.0001) similar to those published previously from this laboratory 33 The vasorelaxant effects of both ANP and CNP were unaffected by pretreatment with ODQ (Fig 4, ANOVA, P<.0001).

Lack of ODQ Effect on 8-Bromo-cGMP-Mediated Relaxation of Phenylephrine-Precontracted Rat Aorta

These experiments tested whether ODQ blocked cGMP-mediated vasorelaxation downstream of guanylyl cyclase. Fig 5 shows that the membrane-permeable cGMP analog 8-bromo-cGMP (10^{-7} to 3x10^{-4} mol/L) resulted in a concentration-dependent relaxation of phenylephrine-contracted rat aortas (IC_{50}=30 μmol/L; ANOVA, P<.0001). ODQ pretreatment of a paired aortic ring in each experi-
More specifically, ODQ completely blocked nonoate effects in the aortic cells and significantly shifted the nonoate concentration-response curve in the COS-7 sGC cells to the right and suppressed the maximal effect. ODQ therefore presented a mixed competitive/noncompetitive antagonism. Nonoate and nitroglycerin, another NO donor, were likewise found to relax phenylephrine-precontracted rat aorta in a concentration-dependent manner. ODQ caused a similar significant rightward shift of the nonoate-response curve and an attenuation of the maximal effect, indicating a competitive/noncompetitive inhibition of the vasorelaxant effect of the NO donor. This result is consistent with the observations on whole-cell cGMP production in this study and a previous study in isolated intact bovine pulmonary arteries.

These data led to a further characterization of ODQ effects on the NO/sGC/cGMP signaling cascade in both the COS-7 sGC cells and rat aortas. ODQ could have resulted in the observed effects on cGMP concentrations by directly inhibiting sGC or lowering donated-NO concentrations by the generation of superoxide anion. Kinetic studies on sGC activity in the cytosolic fraction from COS-7 sGC cells demonstrated that ODQ is a mixed competitive/noncompetitive inhibitor of the enzyme with respect to nonoate stimulation in the presence of fixed concentrations of Mn$^{2+}$ and Mn$^{2+}$-GTP. This result is consistent with the pharmacological activity of ODQ on both nonoate-mediated cGMP production in whole cells and aortic vasorelaxation and with a recent report that ODQ acts in part by binding to the heme-moiey. Additionally, we considered whether the noncompetitive effect of ODQ on sGC activity was mediated by competition with metal ion- or substrate nitrate-donor activity in isolated vessels from wild-type versus sGC-gene knockout animals, if and when the latter become available.

The results of the present study clearly demonstrate that ODQ can inhibit sGC activation independent of the presence of NO donors or NOS activity and that ODQ neither modulates the vascular effects of membrane-permeable cGMP analogs nor auto-oxidizes NO. These observations dissociate the activities of ODQ from effects on NO concentrations and downstream cGMP-mediated cellular events (e.g., cyclic nucleotide-sensitive protein kinases or cyclic nucleotide-mediated activation of ion channels), further supporting a direct effect of the quinoxalin derivative to inhibit the purified enzyme. These data support the hypothesis that sGC mediates vasorelaxant activity associated with NO donors. ODQ will likely become an important tool in attempting to differentiate between sGC-dependent and independent effects of NO.

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