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ACCELERATED COMMUNICATION

Potent and Selective Inhibition of Nitric Oxide-Sensitive Guanylyl Cyclase by 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one

JOHN GARTHWAITE, ERIC SOUTHAM, CAROLINE L. BOULTON, ERIK B. NIELSEN, KURT SCHMIDT, and BERND MAYER

Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, UK (J.G., E.S., C.L.B.), Pharmaceuticals Division, Novo Nordisk A/S, 2760 Måløv, Denmark (E.B.N.), and Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universitat, Universität Graz, A-8010 Graz, Austria (K.S., B.M.)

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SUMMARY

In brain and other tissues, nitric oxide (NO) operates as a diffusible second messenger that stimulates the soluble form of the guanylyl cyclase enzyme and so elicits an accumulation of cGMP in target cells. Inhibitors of NO synthesis have been used to implicate NO in a wide spectrum of physiological and pathophysiological mechanisms in the nervous system and elsewhere. The function of cGMP in most tissues, however, has remained obscure. We have now identified a compound, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), that potently and selectively inhibits NO-stimulated guanylyl cyclase activity. In incubated slices of cerebellum, ODQ reversibly inhibited the NO-dependent cGMP response to glutamate receptor agonists (IC_{50} ~ 20 nM) but did not affect NO synthase activity. The compound did not affect synaptic glutamate receptor function, as assessed in hippocampal slices, nor did it chemically inactivate NO. ODQ did, however, potently inhibit cGMP generation in response to NO-donating compounds. An action on NO-stimulated soluble guanylyl cyclase was confirmed in studies with the purified enzyme. ODQ failed to inhibit NO-mediated macrophage toxicity, a phenomenon that is unrelated to cGMP, nor did it affect the activity of particulate guanylyl cyclase or adenylyl cyclase. ODQ is the first inhibitor that acts selectively at the level of a physiological NO "receptor" and, as such, it is likely to prove useful for investigating the function of the cGMP pathway in NO signal transduction.

The diffusible molecule NO has been implicated in a wide range of physiological functions, including endothelium-dependent relaxation of blood vessels, chemical communication between peripheral nerves and smooth muscle, and long term modifications in the efficacy of central synapses (1, 2). In the central nervous system, NO was originally identified as the endogenous intercellular messenger that is generated in a Ca^{2+}-dependent manner after glutamate (particularly NMDA) receptor activation and that elicits cellular cGMP accumulation in target cells by stimulating soluble guanylyl cyclase (3). The subsequent function of cGMP in this and many other tissues has remained mysterious. Moreover, NO may have effects that are unrelated to soluble guanylyl cyclase stimulation. We have now identified a unique and powerful tool with which to investigate the function of the NO-cGMP pathway, namely a potent and selective inhibitor of NO-sensitive guanylyl cyclase.

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Materials and Methods

cGMP levels and NO synthase activity in brain slices. Cerebellar slices (400-μm thick in the sagittal plane) were cut from immature (8-day-old) or adult (4–6-week-old) Wistar rats (of either gender) (4). In some experiments, transverse hippocampal slices (400-μm thick) from adult rats were used (5). The slices were incubated at 37° in aCSF containing 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4, and 11 mM glucose. cGMP responses to the glutamate receptor agonists NMDA and AMPA and to NO donor drugs were determined as described previously (4, 5). When tested, the compound ODQ (syn-

ABBREVIATIONS: NO, nitric oxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; aCSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; EPSP, excitatory postsynaptic potential; SNP, sodium nitroprusside; ANF, atrial natriuretic factor; NMDA, *N*-methyl-D-aspartate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

thesized by Novo Nordisk) (see Fig. 1A, *inset*, for structure) was added 15–30 min before the effector agent, because pilot experiments demonstrated that its effects were maximal with this preincubation period. NO synthase activity was measured by monitoring the nitroarginine (100 μM)-sensitive conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline (6). Slices were incubated for 30 min in aCSF containing 20 μM L-[^{14}C]arginine (150,000 dpm/ml) and 1 mM L-citrulline, with or without ODQ, and then 100 μM NMDA was added. After 5 min the slices were withdrawn, plunged immediately into boiling 50 mM Tris/4 mM EDTA buffer, pH 7.6, homogenized, and, after removal of L-[^{14}C]arginine by using 50% (by volume) Dowex 50W ion exchange resin (sodium form), the amount of L-[^{14}C]citrulline in the supernatant was quantified using liquid scintillation counting. The same slices were also assayed for their cGMP and protein contents.

Hippocampal slice electrophysiology. Transverse hippocampal slices (400-μm thick) were prepared from adult (4–6-week-old) rat brains using standard techniques, placed in a submersion chamber held at 24°, and perfused with oxygenated aCSF. Field EPSPs, evoked at a frequency of 0.033 Hz, were recorded from stratum radiatum of CA1 after electrical stimulation of the Schaffer collateral-commissural pathway (7). Stable EPSPs (which, under these conditions, are mediated by AMPA receptors) were recorded for at least 20 min before drug application and were quantified by measurement of their slopes. NMDA receptor-mediated EPSPs were evoked in the presence of 10 μM 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*F*)quinoxaline and 50 μM picrotoxin.

Soluble guanylyl cyclase assay. Purified soluble guanylyl cyclase (150 ng) from bovine lung (a kind gift from the late Eycke Böhme, Free University of Berlin, Germany) was incubated at 37° for 10 min in 0.1 ml of 50 mM triethanolamine-HCl buffer, pH 7.4, containing 10 μM [α-^{32}P]GTP (200,000–300,000 cpm), 1 mM Mg^{2+}, 2 mM glutathione, and 1 mM unlabeled cGMP. Reactions were stopped by $ZnCO_3$ precipitation, and [^{32}P]cGMP was isolated (8).

Cyclic nucleotides in endothelial cells. Porcine aortic endothelial cells were isolated by enzymatic treatment with 0.1% collagenase and were cultured in Petri dishes, for up to three passages, in Opti-MEM medium (Gibco-BRL) containing 3% fetal calf serum and antibiotics. Before the experiments cells were subcultured in six-well plastic plates, and confluent monolayers (approximately 10^6 cells/dish) were used for cGMP/cAMP measurements. Endothelial cells were washed twice with isotonic HEPES buffer, pH 7.4, containing 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$ and were preincubated for 15 min at 37° in 1.4 ml of the same buffer containing 1 mM isobutylmethylxanthine and 1 μM indomethacin, in the absence or presence of ODQ. The incubation was started by addition of 0.1 ml of a solution of ANF (human α-ANF_{1-28}), *S*-nitrosoglutathione, or forskolin and was stopped after 4 min by removal of the incubation medium and addition of 1 ml of 0.01 N HCl. Within 1 hr, intracellular cGMP and cAMP were completely released into the supernatant and were measured by radioimmunoassay.

Macrophage toxicity. RAW 264.7 macrophages were cultured in Petri dishes in Opti-MEM medium (Gibco-BRL) supplemented with 3% fetal calf serum and antibiotics. The cells were subcultured in 24-well plates and confluent monolayers were incubated at 37° with L-methylarginine or ODQ, in the absence or presence of 2 μg/ml bacterial lipopolysaccharide and 50 units/ml interferon-γ. After 24 hr, the culture media were assayed for lactate dehydrogenase activity (to quantify cell death) and nitrite ion concentration (to measure NO formation) (9).

NO autoxidation. Autoxidation of NO was determined with an NO-sensitive electrode (World Precision Instruments, Mauer, Germany) as described recently (10). Briefly, 3 μl of a saturated solution of NO gas (~2 mM) were added to 1.8 ml of air-saturated triethanolamine-HCl buffer (50 mM; pH 7.0). The output signal was recorded at a sampling rate of 3 Hz.

Results and Discussion

In cerebellar slices, ODQ inhibited the cGMP response to the glutamate receptor agonist NMDA, with an IC_{50} of about 20 nM (Fig. 1A). This effect of ODQ was fully reversible, because the response to NMDA in slices exposed to 100 nM ODQ for 30 min and then maintained for a 1-hr washout period was 101 ± 15% of controls (mean ± standard error, four experiments). The loss of response produced by ODQ was unlikely to be due to NMDA receptor blockade or to inhibition of NO synthase, because the cGMP-enhancing ef-

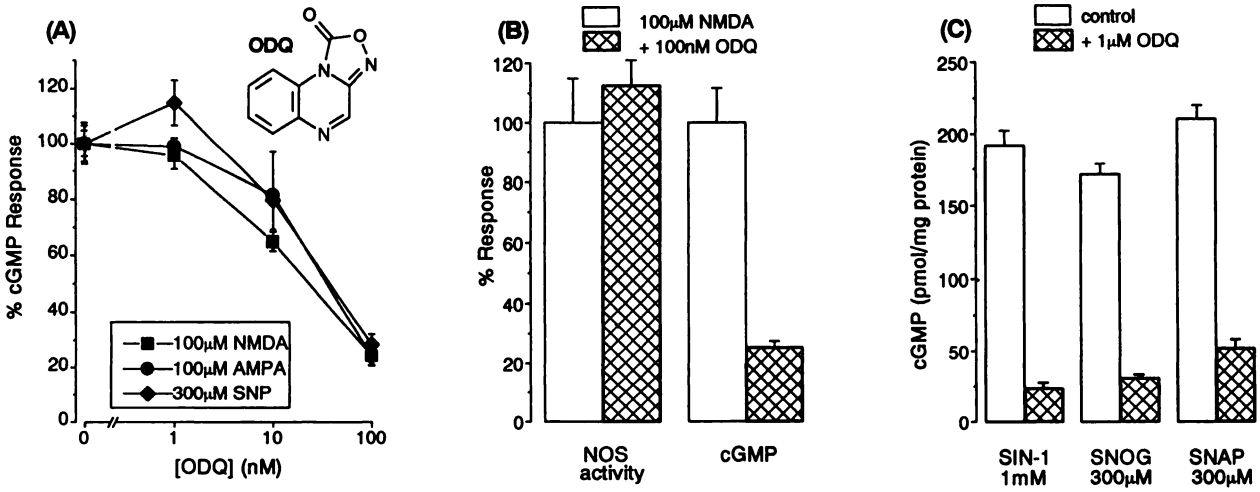

Fig. 1. ODQ potently inhibits NO-stimulated cGMP accumulation, in cerebellar slices, induced by exposure to NMDA, AMPA, or exogenous NO but does not affect NO synthase activity. A, ODQ (structure shown in *inset*) concentration-dependently inhibited cGMP responses in immature cerebellar slices exposed to NMDA (2 min), AMPA (0.5 min), or the NO donor SNP (5 min). The amplitudes of the control responses were as follows: NMDA, 144 ± 10 pmol/mg of protein; AMPA, 11 ± 1 pmol/mg of protein; SNP, 72 ± 1 pmol/mg of protein. Basal cGMP levels were 0.7 ± 0.1 pmol/mg of protein. Each data point represents the mean ± standard error of four slices. B, Incubation of immature cerebellar slices with ODQ had no effect on NO synthase (*NOS*) activity in slices stimulated for 5 min with NMDA (control value, 17 ± 3 pmol/mg of protein/min; five experiments), whereas, in the same slices, it reduced cGMP responses (control value, 171 ± 20 pmol/mg of protein). C, ODQ inhibited cGMP responses to three different NO donors (5-min exposure) in adult cerebellar slices (four slices). *SIN-1*, 3-morpholinosydnonymine; *SNOG*, *S*-nitrosoglutathione; *SNAP*, *S*-nitrosoacetylpenicillamine.

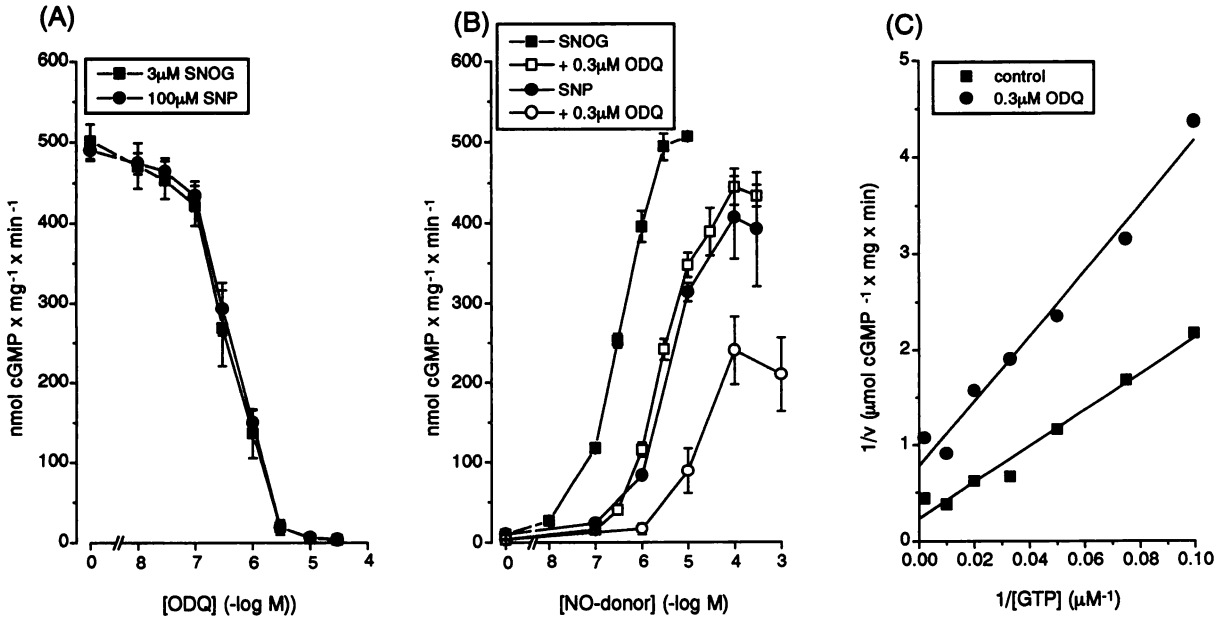

Fig. 2. ODQ inhibits purified soluble guanylyl cyclase. A, Concentration-response curves for ODQ-mediated inhibition of soluble guanylyl cyclase activity induced by 100 μM SNP or 3 μM *S*-nitrosoglutathione (*SNOG*). The data points are means ± standard errors of three separate determinations. B, Concentration-response curves for *S*-nitrosoglutathione and SNP in the absence and presence of 0.3 μM ODQ. The data points are means ± standard errors of three experiments. C, Double-reciprocal plot of soluble guanylyl cyclase activity in the presence of 100 μM SNP and 10–500 μM GTP. The data points are mean values of four separate determinations. After weighting of the data by a standard procedure (19), K_m values in the absence and presence of 0.3 μM ODQ were calculated to be 31 and 35 μM, respectively; the corresponding V_{max} values were 3.0 and 1.3 μmol/mg of protein/min.

fect of the non-NMDA receptor agonist AMPA was similarly inhibited (Fig. 1A), whereas NMDA-stimulated NO synthase activity was not modified (Fig. 1B). In confirmation, at the highest concentration tested (10 μM), ODQ did not affect the activity of purified brain NO synthase.[1]

As in cerebellum, cGMP accumulation in hippocampal slices after exposure to NMDA (300 μM, for 2 min) or AMPA (100 μM, for 30 sec) was nullified by 1 μM ODQ (data not shown); specific tests for an action of ODQ on synaptically activated glutamate receptors were carried out using these tissues. After 30–40-min exposure to ODQ at the highest concentration examined (10 μM), AMPA and NMDA receptor-mediated synaptic potentials were not significantly changed. The values of the initial slopes were 104 ± 6% and 107 ± 5% of controls, respectively (values measured over a 10-min period immediately before the ODQ application; means ± standard errors, four to six experiments).

These findings indicate that ODQ acts downstream from the glutamate receptor-NO synthase pathway. In support of this conclusion, ODQ was able to inhibit cGMP accumulation in cerebellar slices exposed to the NO donor SNP (300 μM), with a potency similar to that shown against NMDA and AMPA (Fig. 1A). Responses to other NO donors were also sensitive to the compound (Fig. 1C), suggesting either that it directly inactivates NO or that it inhibits stimulation of guanylyl cyclase by NO.

The first possibility was eliminated by direct tests using a NO electrode. In accordance with previous reports (11), the third-order rate constant for NO autoxidation was found to be about $9 \times 10^6\ M^{-2} \times sec^{-1}$. ODQ (0.3 mM final concentration) affected neither the amplitude of the response of the electrode to NO nor the autoxidation kinetics of NO (two experiments). In addition, at concentrations up to 10 μM, ODQ did not generate superoxide anions (which rapidly react with NO) either in the presence or in the absence of thiols, as determined by superoxide dismutase-inhibitable reduction of cytochrome *c*.[1]

Experiments to directly test the effect of ODQ on soluble guanylyl cyclase were performed with purified enzyme. ODQ inhibited the enzyme activity evoked by matched concentrations of two different NO donors (SNP and *S*-nitrosoglutathione), with identical inhibition curves (Fig. 2A). Dose-response curves for both NO donors were shifted to the right in the presence of 0.3 μM ODQ, with, at least in the case of SNP, a clear reduction in the maximum effect (Fig. 2B). Further studies are needed to more accurately classify the type of inhibition with respect to NO. Kinetic analysis suggested that the inhibition was noncompetitive with respect to the substrate GTP (Fig. 2C).

To determine whether ODQ acts selectively on soluble guanylyl cyclase or whether it can also inhibit the membrane-associated, or particulate, type of guanylyl cyclase that transduces natriuretic peptide receptor activation, its effects on ANF-induced cGMP accumulation in endothelial cells were explored. ODQ reduced basal cGMP levels with a potency in the nanomolar range, similarly to that observed against the NO donor *S*-nitrosoglutathione (Fig. 3A). However, the increase in cGMP levels induced by 100 nM ANF persisted in the presence of ODQ at concentrations of up to 100 μM (Fig. 3B). cGMP responses to lower ANF concentrations were, likewise, insensitive to ODQ (Fig. 3B). Adenylyl cyclase activity, assayed by measuring cAMP accumulation induced by forskolin, was unaffected by high ODQ concentrations (Fig. 3C).

Evidence that ODQ does not block actions of NO that are

[1] B. Mayer, unpublished observations.

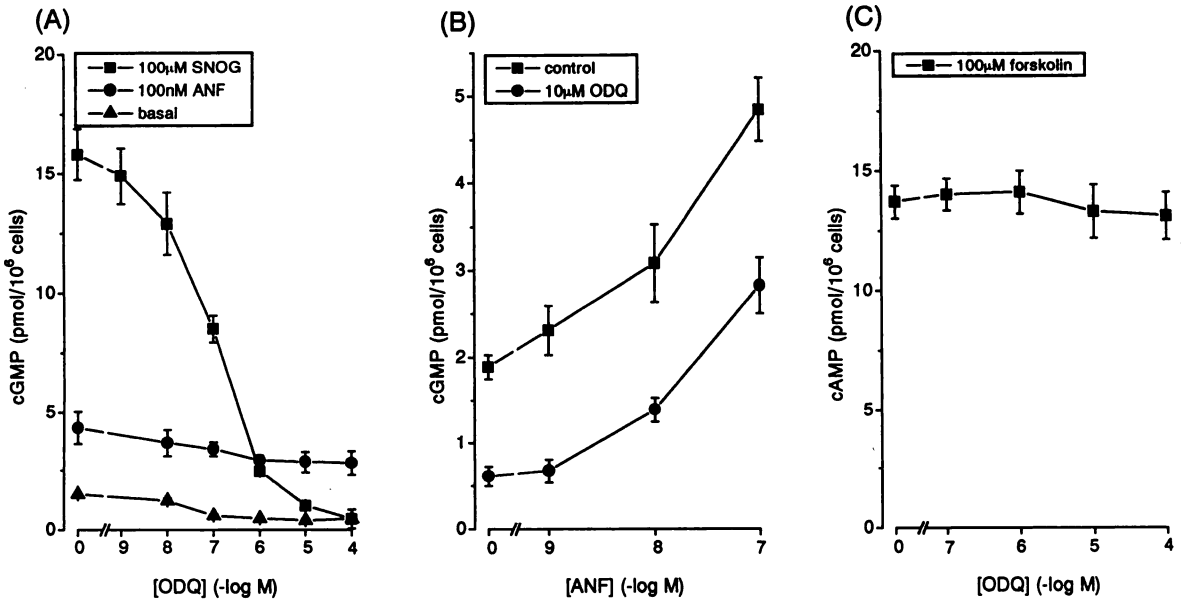

Fig. 3. ODQ does not inhibit ANF-stimulated particulate guanylyl cyclase or adenylyl cyclase. A, In endothelial cells, increasing concentrations of ODQ reduced basal cGMP levels and cGMP accumulation induced by 100 μM *S*-nitrosoglutathione (*SNOG*), without affecting the cGMP response to 100 nM ANF. B, Dose-cGMP response curves for ANF in the absence and presence of 10 μM ODQ are shown. C, In a wide range of concentrations, ODQ failed to affect cAMP accumulation induced by 100 μM forskolin. The data points are means ± standard errors of three to five experiments.

unrelated to guanylyl cyclase activation was obtained by examining the cytotoxicity of activated macrophages. Exposure of macrophages to bacterial lipopolysaccharide and/or cytokines results in the time-dependent expression of the inducible isoform of NO synthase, and the resulting NO ultimately causes cell death through mechanisms that, although not yet clearly defined, include inhibition of metabolic enzymes (1). In our experiments, ODQ failed to influence the cytotoxicity of activated macrophages under conditions where the NO synthase inhibitor L-methylarginine was effective (Table 1). Furthermore, in the same experiments, L-methylarginine simultaneously inhibited NO formation, whereas ODQ did not, suggesting that the inducible NO synthase, like the neuronal Ca^{2+}-dependent isoform, is insensitive to ODQ.

TABLE 1

Effects of L-methylarginine and ODQ on NO-induced macrophage cytotoxicity

Values are means ± standard errors (three experiments).

	Lactate dehydrogenase release	Nitrite concentration
	milliunits/ml	*μM*
Untreated macrophages		
Control	65 ± 5	1.4 ± 0.6
L-Methylarginine, 100 μM	71 ± 4	0.9 ± 0.1
ODQ		
10 μM	67 ± 4	2.6 ± 0.2
100 μM	71 ± 5	2.7 ± 0.2
Induced macrophages		
Control	229 ± 17	24 ± 2
L-Methylarginine		
10 μM	211 ± 30	24 ± 4
100 μM	84 ± 3	17 ± 3
1 mM	51 ± 4	11 ± 1
ODQ		
1 μM	247 ± 10	24 ± 2
10 μM	249 ± 8	24 ± 3
100 μM	302 ± 1	27 ± 1

These results demonstrate that ODQ is a potent inhibitor of NO-stimulated soluble guanylyl cyclase activity, without actions on particulate guanylyl cyclase or on adenylyl cyclase. In brain slices stimulated with glutamate receptor agonists, ODQ does not appear to interfere with any of the steps leading to NO synthesis; moreover, it does not inhibit constitutive or inducible NO synthase activity and it does not inactivate NO.

Two other compounds are often referred to, and used, as guanylyl cyclase inhibitors, namely LY-83583 and methylene blue. In reality, LY-83583 was originally reported to stimulate guanylyl cyclase activity but, paradoxically, to lower tissue cGMP levels nonspecifically and by an unknown mechanism (12). Although under certain assay conditions guanylyl cyclase inhibition has been observed (and attributed to a metabolite), LY-83583 more potently inhibits NO release (13) and has been shown to have several other effects (14–16) that, collectively, make any results obtained with this chemical very difficult to interpret. Methylene blue is actually a weak guanylyl cyclase inhibitor that more effectively generates superoxide anions and inhibits NO synthase (17).

ODQ thus represents the first inhibitor acting on the NO "receptor," soluble guanylyl cyclase, although further work will be required to define more precisely its mechanism of action. In the brain, both the NO synthetic machinery and NO-stimulated cGMP accumulation display a widespread and correlated distribution, suggesting a general functional partnership between the two (18). The function of cGMP, which has been known for a quarter of a century to be present in the brain, has remained elusive. ODQ represents a powerful and unique tool that we anticipate will play a major role in further elucidating the physiological significance of the NO-cGMP pathway, not only in the nervous system but also

in many other organs in which this signaling system is present.

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Send reprint requests to: John Garthwaite, Wellcome Research Laboratories, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS, UK.