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Pharmacology

cGMP mediates the vascular and platelet actions of nitric oxide: Confirmation using an inhibitor of the soluble guanylyl cyclase

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ABSTRACT **The L-arginine:nitric oxide (NO) pathway is believed to exert many of its physiological effects via stimulation of the soluble guanylyl cyclase (SGC); however, the lack of a selective inhibitor of this enzyme has prevented conclusive demonstration of this mechanism of action. We have found that the compound 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ) inhibits the elevation of cGMP induced by the NO donor *S*-nitroso-DL-penicillamine in human platelets and rat vascular smooth muscle (IC_{50} = 10–60 nM and <10 nM, respectively) and that this is accompanied by prevention of the platelet inhibitory and vasodilator actions of NO donors. ODQ also inhibited the antiaggregatory action of NO generated by the platelets but did not affect the action of prostacyclin or that of a cGMP mimetic. In addition, ODQ inhibited the vasodilator actions of endogenously released NO and of NO generated after induction of NO synthase in vascular preparations. It did not, however, affect the increase in vascular smooth muscle cGMP or the dilatation induced by atrial natriuretic factor. ODQ had no effect on NO synthase activity, nor did it react with NO. It did, however, potently ($IC_{50} \approx 10$ nM) inhibit the activity of the SGC in cytosol obtained from crude extract of rat aortic smooth muscle. Thus ODQ prevents the actions of NO on platelets and vascular smooth muscle through its potent inhibitory effect on the SGC.**

Nitric oxide (NO), *S*-nitrosothiols, and other NO donors (1) activate the soluble guanylyl cyclase (SGC) by binding to the heme moiety of the enzyme (2). This results in increased cGMP concentrations that are associated with relaxation in vascular tissue (for review, see ref. 3). In addition, these compounds increase cGMP in platelets, and this is thought to be the mechanism by which they inhibit platelet function (4, 5).

Studies attempting to investigate the role of SGC in the mediation of the physiological actions of NO have been hampered by the lack of a potent and selective inhibitor of this enzyme. Methylene blue has been used as such; however, this compound also inhibits prostacyclin (PGI_2) production (6, 7), generates superoxide anions (8–10), and directly inhibits NO synthase (11). Furthermore, LY 83583 (6-anilino-5,8-quinolinedione), which inhibits elevation of cGMP concentrations in a number of tissues (12, 13), probably does so by generating superoxide anions (14).

1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) has been shown to inhibit *N*-methyl-D-aspartate-induced increases in cGMP concentrations in rat brain slices and to inhibit the activity of SGC purified from bovine lung (15). We have now used this compound to characterize the role of SGC in the actions of NO in platelets and vascular tissue.

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MATERIALS AND METHODS

Washed Platelet Suspensions. Human blood was collected, and PGI_2-washed platelet (WP) suspensions were prepared as described (16).

Platelet cGMP Concentrations. WPs (2–2.5 × 10^8/ml) were incubated without stirring in a platelet-ionized calcium lumiaggregometer (Chronolog) for 20 min at 37°C in the absence or presence of ODQ (0.1–1 μM). They were then incubated, with stirring, either alone or in the presence of *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; 1–100 μM) for 4 min. The samples were frozen and maintained at −80°C. The cGMP concentrations in disrupted platelets were determined by enzyme immunoassay as described (17).

Vascular Smooth Muscle cGMP Concentrations. Rat aortic rings were isolated, denuded of endothelium, and allowed to equilibrate for 2 h in Krebs solution containing indomethacin (5 μM). The medium was changed after every hour. The buffer was then replaced with fresh buffer containing the selective cGMP phosphodiesterase inhibitor zaprinast (3 μM) in the presence or absence of ODQ (0.3–1 μM). After a further 40 min, SNAP (0.2 μM) or atrial natriuretic peptide (ANP; 100 nM) was added to some preparations for 1 min, and the tissue was rapidly frozen in liquid nitrogen. The frozen tissues were homogenized using a stainless steel pestle and mortar in dry ice. The homogenate was then incubated in 1 ml of ice-cold perchloric acid (0.5 M) at 4°C for 1 h. After centrifugation at 12,000 × *g* for 5 min at 4°C and neutralization with K_3PO_4 (1 M), cGMP concentrations in the supernatant were measured using a specific enzyme immunoassay kit (Amersham). The soluble protein concentration of each sample was measured by the Coomassie blue binding method using Bio-Rad protein reagent (Bio-Rad) with bovine serum albumin as a standard.

Platelet Aggregation. Platelet aggregation was measured in a platelet-ionized calcium lumiaggregometer. ODQ (0.001–3 μM) was incubated with WPs (2–2.5 × 10^8/ml) for 1–30 min at 37°C prior to the addition of collagen (0.1–10 μg/ml). *S*-nitroso-DL-penicillamine (SNPL; 1–3 μM), SNAP (0.01–100 μM), or PGI_2 (10 nM) were incubated with WPs for 1 min prior to the addition of collagen (0.3–5 μg/ml), and their effects on platelet aggregation in the presence or absence of ODQ were studied for 3 min. In some experiments, a membrane-permeable stable analogue of cGMP, 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP; 1 mM) was incubated with WPs for 30 min in the presence or absence of ODQ (1 μM) prior to the addition of collagen (1–3 μg/ml).

Abbreviations: ODQ, 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one; SGC, soluble guanylyl cyclase; PGI_2, prostacyclin; WP, washed platelet; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; ANP, atrial natriuretic peptide; SNPL, *S*-nitroso-DL-penicillamine; SNP, sodium nitroprusside; Ach, acetylcholine; GSNO, *S*-nitrosoglutathione; GTN, glyceryl trinitrate; PE, phenylephrine; LPS, lipopolysaccharide; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate.
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Cascade Bioassay. The cascade bioassay was performed as described (18). Briefly, a segment (6 cm) of the rabbit thoracic aorta with intact endothelium (donor tissue) was removed, cleaned, and perfused intraluminally with Krebs buffer. Three spiral strips of rabbit endothelium-denuded aorta arranged in a cascade (detector tissues) were superfused with the effluent from the donor aorta. The detector tissues were contracted submaximally with 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{1\alpha}$ (U46619; 30 nM). The release of NO from the donor tissue was stimulated with acetylcholine (ACh; 1 μM). Atropine sulfate (0.2 μM) was infused over the detector tissues to block any direct action of ACh. The amplification of the recorder (Graphtec, Linear Corder Mark VII WR3101) was adjusted so that similar relaxations to the same concentration of glyceryl trinitrate (GTN; 0.03–1 μM) were observed in each detector tissue. ODQ (0.0001–1 μM) was infused over the detector tissues for 30 min, after which time its effects on the relaxation induced by NO released from the donor tissue and by an NO donor, *S*-nitrosoglutathione (GSNO; 0.02–6 μM), were investigated. The responses were expressed as a percentage of the relaxation to GTN. The sensitivity of the bioassay tissues to GTN remained unchanged for the duration of the experiments.

Organ Bath Studies. Rings of rat thoracic aorta, with and without endothelium, were prepared, and their tone was studied as described (19). The effect of ODQ on the relaxation caused by NO formed by the constitutive endothelial NO synthase was determined in endothelium-intact rings. The integrity of the endothelium was assessed by obtaining cumulative relaxation curves to ACh (0.005–2.56 μM) in rings precontracted submaximally (EC_{90} = 280 nM) with phenylephrine (PE). After washout, the rings were recontracted with a threshold concentration of PE (EC_{10} = 8–24 nM). A cumulative concentration curve to ODQ (0.0003–3 μM) was then obtained. The tissues were then further contracted with PE to the EC_{90} (without washout), and a cumulative relaxation curve to ACh (0.005–2.56 μM) was repeated in the presence of ODQ (3 μM).

The effect of ODQ on ANP- and isoprenaline-induced relaxation was examined in endothelium-intact aortic rings. The tissues were precontracted with PE (EC_{90}), and the relaxation response to ANP (0.1–100 nM) or isoprenaline (0.1–300 nM) was assessed in the absence or presence of ODQ (1 μM).

In another set of experiments, endothelium-denuded rings were used to study the effect of ODQ on the relaxation induced by NO formed by the inducible NO synthase of the vascular smooth muscle. Briefly, lipopolysaccharide (LPS; 10 ng/ml) was added to endothelium-denuded aortic rings, some of which were pretreated for 10 min with ODQ (3 μM). The rings were then contracted submaximally with PE (ED_{90} = 100 nM). The change in tone in the presence or absence of ODQ (3 μM) was monitored for 6 h.

In separate experiments, endothelium-denuded rings were pretreated with LPS, contracted with PE (ED_{90}), and left for 6 h before studying the responses induced by cumulative concentrations of ODQ (0.003–3 μM).

For data analysis, the relaxations and contractions of rings were expressed as a percentage of the tone induced by PE.

Cytosolic Extract of SGC. Three rat aortae without endothelium were frozen in liquid nitrogen and homogenized with a stainless steel pestle and mortar in dry ice. The homogenized tissue was dissolved in 1.5 ml of buffer containing 50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, and 250 mM sucrose (pH 7.4) and centrifuged (Beckman centrifuge) at 105,000 $\times$ *g* for 60 min at 4°C. The supernatant was used as the source of SGC in the assay and for determination of soluble protein concentration. Enzyme activity was assessed in 1.5-ml Eppendorf tubes containing 190 μl of assay buffer (25 mM Tris, 10 μM–5 mM GTP, 5 mM $MgCl_2$, 3 mM 3-isobutyl-1-methylxanthine, 1 mM EGTA, and 100 μM sodium nitroprusside (SNP) at pH 7.4) and either 10 μl of assay buffer for control or 10 μl of ODQ solutions giving final concentrations ranging between 1 nM and 10 μM at 37°C. The reaction was started by addition of 10 μl of supernatant and terminated by addition of 20 μl of 20% perchloric acid and by rapid freezing in liquid nitrogen after 1, 3, and 10 min. To obtain a zero time point value, perchloric acid was added before the supernatant. The samples were then neutralized with an equal volume of 1 M K_3PO_4 and centrifuged at 12,000 $\times$ *g* for 5 min at 4°C. The cGMP concentrations in the supernatant were measured by a specific enzyme immunoassay kit (Amersham, UK). The soluble protein concentration in the parent supernatant was measured by the Coomassie blue binding method using Bio-Rad protein reagent (Bio-Rad, Germany) with bovine serum albumin as a standard.

Electrochemical Measurement of NO. NO gas (1–10 μM) or SNAP (1–50 μM) was added to Tyrode's solution (pH 7.2, 37°C) in the presence or absence of ODQ (1–10 μM). NO gas in solution was measured electrochemically using a Clark-type NO-sensitive electrode (Diamond General Development Corporation, MI, USA). The electrode was calibrated with anaerobic solutions of pure NO gas in deoxygenated water (20). $CuSO_4$ (5 μM) was used to increase the release of NO from SNAP (21). These electrodes are highly selective for NO and do not detect nitrite, nitrate, peroxynitrite, nitroxyl, superoxide, or hydrogen peroxide (22).

Platelet NO Synthase Activity. Platelets (10^9/ml) were homogenized by freeze-thawing (three cycles in liquid nitrogen followed by thawing at 37°C) and centrifuged at 10,000 $\times$ *g* for 5 min at 4°C. The NO synthase activity in the soluble fraction was assessed by measuring the formation of [^{14}C]citrulline from L-[U-^{14}C]arginine (150,000 dpm/100 μl) (23) in the presence or absence of ODQ (0.1–10 μM).

Interactions Between ODQ and Oxyhemoglobin. Human oxyhemoglobin (18.5 μM), prepared according to the method of Paterson *et al.* (24), was incubated with ODQ (5–50 μM) at 20°C. The formation of methemoglobin was monitored by measuring the increase in absorbance at 630 nm. Initial rates of methemoglobin formation were calculated during the first 10 min of the reaction. In some experiments the visible absorption spectra between 500 and 700 nm were measured every minute during incubation of oxyhemoglobin with ODQ. The second-order rate constant for this reaction was calculated from the linear plot of the initial rate of oxidation as a function of ODQ concentration.

Reagents. ODQ (Novo Nordisk, code NNC07-9008; 1 mM) was dissolved in 50% dimethyl sulfoxide and diluted in isotonic saline or MilliQ water. The bromide salt of ACh, PE, isoprenaline, ANP, DL-dithiothreitol, EDTA, EGTA, GTP, 3-isobutyl-1-methylxanthine, and zaprinast were purchased from Sigma. *Salmonella typhosa* LPS was from Difco; SNPL was a kind gift of S. Askew and A. Butler (University of St Andrews, Scotland); SNAP, GSNO, and the sodium salt of PGI_2 were obtained from Wellcome. U46619, GTN, collagen, and perchloric acid were purchased from Calbiochem, DuPont Pharmaceuticals, Hormon-Chemie, and Analar, respectively. 8-pCPT-cGMP was from Biolog, and L-[^{14}C]arginine, and cGMP enzyme immunoassay were obtained from Amersham.

Statistics. Results are the mean $\pm$ SEM of at least three separate experiments. The IC_{50} values for ODQ were calculated using MicroCal ORIGIN 2.8 (MicroCal Software). Student's unpaired *t* test was used to determine the significance of differences between means, and $P < 0.05$ was considered as statistically significant.

RESULTS

Effect of ODQ on Intraplatelet cGMP Levels. The basal platelet cGMP concentration (0.14 $\pm$ 0.06 pmol/10^8 platelets;

$n = 6$) was not affected by ODQ (1 μM; 0.13 ± 0.11 pmol/10^8 platelets; $P > 0.05$; $n = 3$). Incubation of platelets for 4 min with SNAP (1 μM) increased the cGMP concentration to 5.7 ± 1.3 pmol/10^8 platelets ($P > 0.05$; $n = 6$). This increase in cGMP was inhibited by ODQ (0.1–1 μM) in a concentration-dependent manner (0.16 ± 0.13 pmol/10^8 platelets at 1 μM ODQ; $n = 3$; $P < 0.05$, Fig. 1*A*). The effect of ODQ (0.1 μM; 0.89 ± 0.19 pmol/10^8 platelets; $n = 6$) was only partially reversed by a high concentration (100 μM) of SNAP (2.5 ± 0.2. pmol/10^8 platelets; $n = 3$; Fig. 1*A*).

Effect of ODQ on Vascular Smooth Muscle cGMP Levels. The basal concentrations of cGMP in vascular smooth muscle from rat aorta without endothelium (0.48 ± 0.14 pmol/mg of protein) were not affected (0.39 ± 0.11 pmol/mg of protein) by the presence of 3 μM ODQ ($P > 0.05$; $n = 6$). SNAP (0.2 μM) and ANP (100 nM) raised the concentrations of cGMP to 68.8 ± 42.6 and 4.9 ± 0.5 pmol/mg of protein, respectively ($n = 3$ for each). ODQ (0.3 μM) inhibited the elevation of cGMP induced by SNAP (0.75 ± 0.18 pmol/mg of protein; $P < 0.05$; $n = 3$); however, even at 3 μM it did not affect the smaller increase induced by ANP (3.7 ± 0.8 pmol/mg of protein; $P > 0.05$; $n = 3$) (Fig. 1*B*).

Effect of ODQ on Platelet Aggregation and Its Inhibition. Addition of collagen (0.1–10 μg/ml) to WPs resulted in a concentration-dependent platelet aggregation that was enhanced when platelets were preincubated with 3 μM ODQ. The enhancing effect of ODQ was significant ($n = 3$; $P < 0.05$) at concentrations of collagen (0.3, 0.6, and 1 μg/ml) that produced submaximal aggregating effects, so that 10% ± 3%, 25% ± 1%, and 62% ± 6% of light transmission were obtained in the absence and 21% ± 3%, 58% ± 6%, and 88% ± 7% were obtained in the presence of ODQ.

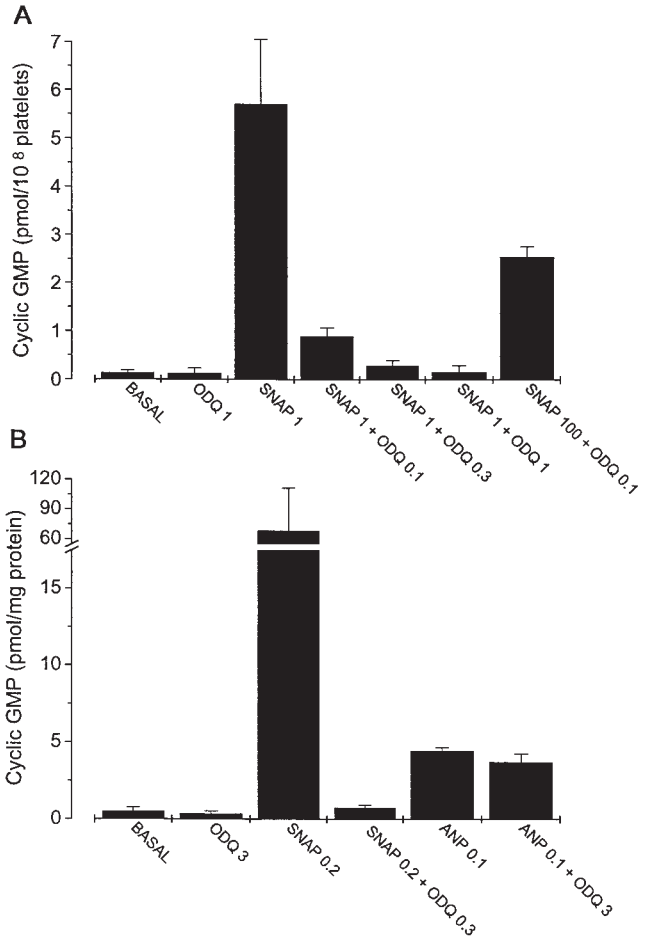

FIG. 1. (*A*) Inhibition by ODQ (0.1–1 μM) of the increase in intraplatelet cGMP concentrations induced by 1 μM SNAP and its partial reversal by a higher concentration (100 μM) of this NO donor. Data are mean ± SEM, $n = 3$–6. (*B*) Inhibition by ODQ (0.3–3 μM) of the increase in vascular smooth muscle cGMP concentrations induced by SNAP (0.2 μM) but not of that caused by ANP (100 nM). Data are mean ± SEM, $n = 3$–6.

SNPL and SNAP (3 μM) each produced a similar, maximally effective (≈90%) inhibition of collagen (1–5 μg/ml)-induced platelet aggregation. Preincubation of WPs with ODQ (0.001–3 μM) for 20–30 min resulted in a concentration-dependent reversal of the inhibition caused by these compounds (see Fig. 2*A* for effect on SNPL), with EC_{50} values of 0.17 ± 0.03 and 0.12 ± 0.08 μM for SNPL and SNAP, respectively. The effect of ODQ (1 μM) was time-dependent and increased from 20% after 10 min to 85–100% after 20–30 min of incubation at 37°C. Furthermore, this effect was reversed, in a concentration-dependent manner, by increasing concentrations of SNAP (maximal reversal of 78% ± 7% at 100 μM; $n = 3$; data not shown).

PGI_2 (10 nM) also inhibited collagen-induced platelet aggregation, an action that was not affected by ODQ (3 μM; Fig. 2*B*). The aggregation induced by collagen (1–3 μg/ml) was maximally inhibited by 8-pCPT-cGMP (1 mM) in the absence or presence of ODQ (1 μM) ($n = 3$; data not shown).

Effect of ODQ on Vascular Relaxation. ODQ (0.0001–1 μM) inhibited, in a concentration-dependent manner, the relaxation of rabbit aortic strips produced by NO released by ACh from the rabbit perfused aorta (Fig. 3*A*). GSNO (0.2–6 μM) elicited a concentration-dependent relaxation of endothelium-denuded rabbit aortic strips that was inhibited by ODQ (1 μM; Fig. 3*B*).

ODQ (0.001–3 μM) induced a concentration-dependent contraction (EC_{50} = 0.24 ± 0.05 μM) of rat endothelium-intact isolated aortic rings precontracted with PE (EC_{10}) in an organ bath (data not shown). No contractile action of ODQ was observed in endothelium-denuded aortic rings ($n = 3$). In addition, ACh (0.005–2.56 μM)-induced relaxation of rat isolated aortic rings was inhibited, but not completely abolished, in the presence of ODQ (3 μM; Fig. 3*C*). In contrast, ANP (0.1–100 nM)-induced relaxation was not affected significantly by 1 μM ODQ (EC_{50} = 3.7 ± 1.4 nM in the absence and 4.2 ± 1.3 nM in the presence of ODQ; $n = 3$; $P > 0.05$), nor did ODQ affect the relaxation induced by isoprenaline (0.1–300 nM) (EC_{50} = 8.0 ± 1.9 nM in the absence and 9.8 ± 2.1 nM in the presence of ODQ; $n = 3$; $P > 0.05$) (data not shown).

LPS (10 ng/ml), when incubated for 6 h with rat isolated endothelium-denuded aortic rings precontracted with PE (100 nM), produced a time-dependent loss of tone in the tissue. This effect was completely prevented by the presence of 3 μM ODQ (Fig. 3*D*). ODQ (0.0003–1 μM) also caused a concentration-dependent contraction (EC_{50} = 0.16 ± 0.03 μM; $n = 3$) of rings relaxed after incubation with LPS for 6 h (data not shown).

Effect of ODQ on Cytosolic Extract of SGC. In the presence of different concentrations of GTP (10 μM–5 mM), cGMP production by the cytosolic extract in response to 100 μM SNP was linear with time (0–10 min) in the absence and presence of ODQ (300 nM) (correlation coefficient = 0.99 for each). The basal cGMP concentration at zero time in the presence of 5 mM GTP and 100 μM SNP was 1.8 ± 0.5 pmol/mg of protein. The cGMP concentration after 10 min in the same conditions was 8428 ± 797 pmol/mg protein ($n = 3$, $P < 0.001$).

The V_{max} values in the absence and presence of ODQ (300 nM) were 525.0 ± 43.6 pmol cGMP per mg of protein per min and 149.7 ± 14.4 pmol cGMP per mg of protein per min, respectively ($n = 3$; $P < 0.0005$). The K_m values in the absence and presence of ODQ (300 nM) were 46.3 ± 22.4 μM and 68.3 ± 34.3 μM, respectively ($n = 3$; $P > 0.05$). ODQ

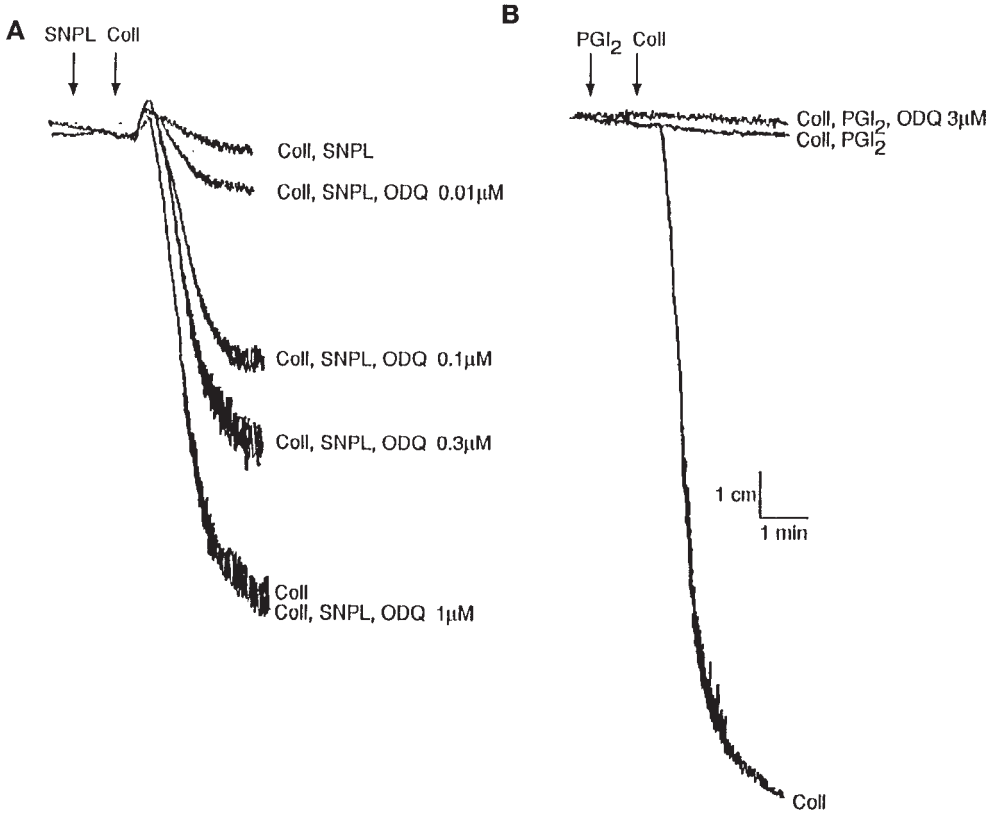

FIG. 2. Effect of ODQ on the inhibition by SNPL (*A*) and PGI_2 (*B*) of collagen-induced platelet aggregation in washed platelets. (*A*) ODQ (0.01–1 μM; Coll, SNPL, ODQ 0.01–1 μM) reversed the inhibitory effect of 3 μM SNPL (Coll, SNPL) on collagen-induced platelet aggregation (Coll). (*B*) ODQ (3 μM; Coll, PGI_2, ODQ 3 μM) did not affect the inhibition by 10 nM PGI_2 (Coll, PGI_2) of collagen-induced platelet aggregation (Coll).

(1 nM–10 μM) inhibited dose dependently the cGMP production by the cytosol in response to 100 μM SNP in the presence of 10 μM GTP. The IC_{50} for ODQ was 9.47 ± 0.52 nM (n = 3).

When ANP (300 nM) was added instead of SNP into the assay buffer, ANP did not increase the cGMP concentration significantly either in the absence (2.0 ± 0.4 pmol per mg of protein per min; n = 3) or presence (1.8 ± 0.4 pmol per mg of protein per min; n = 3; $P > 0.05$) of ODQ (300 nM).

Effect of ODQ on NO and NO Synthase Activity. ODQ (1–10 μM), when added to solutions containing NO (1–10 μM) or SNAP (1–50 μM), affected neither the peak con-

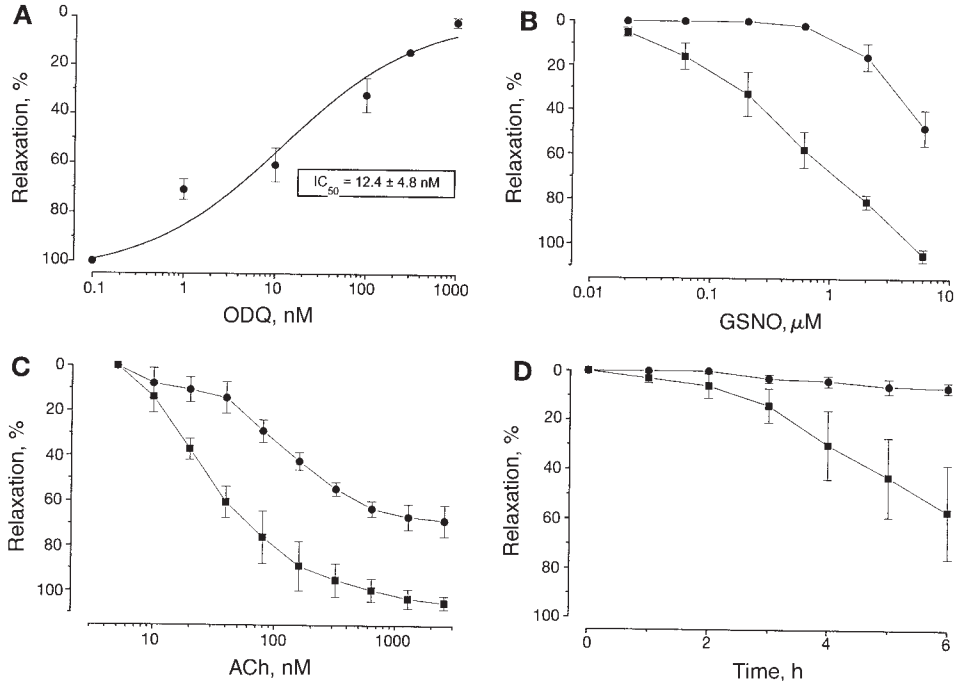

FIG. 3. Effects of ODQ on vascular relaxations. (*A* and *B*) Inhibition by ODQ of the relaxing effect on rabbit aortic strips of NO released by ACh (1 μM) from rabbit perfused aorta (*A*) or from GSNO (*B*). GSNO was infused in the presence (●) or absence (■) of 1 μM ODQ. Data were obtained from the top detector tissue of the cascade bioassay. (*C*) Inhibition by ODQ (3 μM; ●) of ACh-induced relaxation of rat aortic rings (■). (*D*) Inhibition by ODQ (3 μM; ●) of the relaxing effects of NO generated during prolonged incubation of rat vascular rings with LPS (■). Data are mean ± SEM, n = 3.

centration nor the subsequent decomposition of NO ($n = 3$) when measured electrochemically with an NO electrode. The peak concentration (1–2 min) of NO released from 50 μM SNAP was 23.8 ± 1.5 μM in the absence of ODQ and 23.8 ± 0.1 μM in the presence of 10 μM ODQ.

The activity of NO synthase in platelets was 6.2 ± 1.8 pmol of citrulline per mg of protein per min and was not affected significantly (6.8 ± 1.2 pmol of citrulline per mg of protein per min) by 3 μM ODQ ($n = 3$; $P > 0.05$).

Interactions Between ODQ and Oxyhemoglobin. Incubation of ODQ (5–50 μM) with oxyhemoglobin resulted in the conversion to methemoglobin, as evidenced by the characteristic time-dependent decrease in the visible absorption of oxyhemoglobin at 542 and 577 nm and the concomitant increase in absorbance at 630 nm. The second-order rate constant for the reaction between ODQ and oxyhemoglobin (18.5 μM), calculated from the plot of the initial rate of methemoglobin formation as a function of ODQ concentration, was found to be 1133 ± 165 $M^{-1}{\cdot}s^{-1}$ ($n = 4$; mean ± SD).

DISCUSSION

Our results show that ODQ abolishes the increases in cGMP induced by NO donors in platelets and in vascular tissue without affecting basal cGMP concentrations in either preparation. This action of ODQ is dose-dependent, persistent, and only partially overcome by an excess (100:1) of NO donor. ODQ does not react directly with NO itself and has no effect on the release of NO from an NO donor, nor does it affect the activity of NO synthase. Furthermore, ODQ did not prevent the increase in cGMP induced in vascular tissue by ANP. All these results, together with our demonstration that ODQ inhibits the SGC directly, indicate that the actions of ODQ not only in brain (15) but also in platelets and vascular tissue are confined to an inhibitory action on this enzyme.

The antiaggregating action of the NO donors SNPL and SNAP were prevented by treatment with ODQ. This effect was time- and concentration-dependent and, unlike that on cGMP concentrations, could largely be reversed by increasing concentrations of SNAP. ODQ had no effect on the platelet-inhibitory actions of PGI_2, which acts by elevating cAMP concentrations (25), or of 8-pCPT-cGMP, which mimics the actions of cGMP (26, 27).

These results show that ODQ is a potent and selective inhibitor of the SGC and would therefore be a useful tool to investigate hypotheses suggesting that the platelet-inhibitory effects of NO may involve mechanisms other than activation of the SGC. These include inhibition by NO of the low K_m cAMP phosphodiesterase (28), inhibition of glyceraldehyde-3-phosphate dehydrogenase (29–31), ADP ribosylation (32), and increases in $[Ca^{2+}]_i$ independent of SGC (33, 34). In our experiments, high concentrations of SNAP (100 μM) reversed the effect of ODQ on platelet aggregation, but they only partially restored cGMP concentrations, suggesting either that a relatively small increase in cGMP is enough to inhibit platelet aggregation or that the platelet-inhibitory action of supramaximal concentrations of NO may indeed include some cGMP-independent actions.

ODQ also inhibits the vasodilator actions of NO whether it is released basally, following stimulation, or generated by an NO donor (GSNO) or by inducible NO synthase. In contrast, the relaxing effects of ANP and isoprenaline, which act via stimulation of the particulate guanylate cyclase (35) and adenylate cyclase (36), respectively, were not affected by ODQ. ODQ itself caused a concentration-dependent contraction of rat endothelium-intact, but not endothelium-denuded, isolated aortic rings, indicating the presence of a basal cGMP-dependent dilator tone, presumably due to continuous release of NO by the endothelial NO synthase.

ODQ abolished the relaxing effect on rabbit aortic strips of NO released by ACh from a donor aorta; however, even the highest concentration of ODQ used only partially inhibited the direct endothelium-dependent relaxing activity of ACh on rat isolated aortic rings. This suggests either that some of the vasodilator action of ACh is independent of NO or that NO has some dilator action that is independent of cGMP. Interestingly, however, NO synthase inhibitors do cause complete inhibition of this relaxation (19). cGMP-independent mechanisms such as membrane hyperpolarization have been implicated in the mechanism of the vasodilator action of NO (37). In this context, methylene blue has been used in an attempt to demonstrate that NO directly activates Ca^{2+}-dependent K^+ channels in vascular smooth muscle (38). Such experiments should now be repeated in the presence of ODQ to clarify this point.

The effectiveness of ODQ in restoring vascular tone in endotoxin-treated rat vascular rings would suggest that ODQ-like compounds could be of therapeutic use in sepsis, as has been shown for NO synthase inhibitors (39, 40). However, a concomitant inhibition of the platelet SGC may render platelets proaggregatory and thus exacerbate the platelet activation and thrombocytopenia often associated with septicemia (41, 42). Moreover, we have shown that oxyhemoglobin reacts with ODQ to form methemoglobin, and this moderately rapid reaction is likely to inactivate ODQ significantly, given the high concentration of oxyhemoglobin in blood.

ODQ inhibited the activity of a cytosolic extract of SGC obtained from rat vascular smooth muscle, with an IC_{50} comparable to those obtained in platelets and vascular tissue *in vitro*. The V_{max} value for this enzyme activity was reduced in the presence of ODQ, whereas the K_m was unaltered, suggesting that the inhibition was noncompetitive with respect to the substrate, GTP, in agreement with the results obtained by Garthwaite *et al.* (15). Further studies, using purified SGC, will be required to characterize in more detail the inhibitory action of ODQ on this enzyme.

In summary, we have demonstrated that ODQ potently inhibits NO-induced increases in cGMP in platelets and vascular tissue. This, coupled with the fact that it directly inhibits NO-induced activation of SGC, suggests that this compound will be a useful tool to investigate the biological activities of the L-arginine:NO pathway in these and other tissues.

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