

Role of Soluble Guanylyl Cyclase in the Relaxations to a Nitric Oxide Donor and to Nonadrenergic Nerve Stimulation in Guinea Pig Trachea and Human Bronchus¹

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ABSTRACT

The effect of the novel, selective, soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolol[4,3-a]quinoxalin-1-one (ODQ) on the nitric oxide component of the nonadrenergic, noncholinergic relaxation in guinea pig trachea was examined. Relaxant responses to field stimulation (1-16 Hz, 8 V, 1 ms for 15 s) in the presence of indomethacin (3 μ M), atropine (1 μ M), propranolol (1 μ M), α -chymotrypsin (2 U/ml) and histamine (3 μ M) were partially inhibited by 0.1 μ M ODQ and almost abolished by 1 μ M ODQ. In addition, relaxations to the nitric oxide donor 3-morpholinosyndnonimine-N-ethylcarbamide were partially inhibited by 0.1 μ M ODQ and abolished by 1 μ M ODQ. Relax-

ations to 3-morpholinosyndnonimine-N-ethylcarbamide in human bronchus were also substantially inhibited by ODQ (1-10 μ M). By contrast, relaxations elicited by the stable 3',5'-cyclic monophosphate analog 8-bromoguanosine-3',5'-cyclic monophosphate and by isoproterenol were unaffected by 1 μ M ODQ in guinea pig trachea and by 10 μ M ODQ in human bronchus. These results suggest that relaxant responses to endogenously released or exogenously added nitric oxide in guinea pig trachea and human bronchus are mediated *via* the activation of soluble guanylyl cyclase and the formation of guanosine-3',5'-cyclic monophosphate.

Both NO and VIP have been shown to be NANC relaxant transmitters in guinea pig trachea (Ellis and Farmer, 1989; Tucker *et al.*, 1990). The NO component is inhibited by NO synthase inhibitors such as L-arginine analogs (Tucker *et al.*, 1990), whereas the VIP component is inhibited by the peptidase α -chymotrypsin (Ellis and Farmer, 1989). NO has also been demonstrated to be the primary relaxant transmitter in human airways (Ellis and Undem, 1992; Belvisi *et al.*, 1992; Bai and Bramley, 1993). There has been considerable debate about the second-messenger pathway(s) involved in the response to NO in airway smooth muscle. Depending on the species used and on the NO-donor compound studied, arguments have been raised for and against the involvement of soluble guanylyl cyclase in mediating the NO response. Research in this area has been hampered by the lack of selective, potent inhibitors of soluble guanylyl cyclase. Many studies have used the compounds LY 83583 and methylene blue, but these compounds are very weak inhibitors and have many other effects, including inhibition of NO synthase (Mayer *et al.*, 1993) and free radical production (Wolin *et al.*,

1991; Kontos and Wei, 1993), that make the interpretation of results difficult. Recently, however, a new potent, selective inhibitor of soluble guanylyl cyclase, ODQ, has been identified (Garthwaite *et al.*, 1995; Moro *et al.*, 1996). In the present study, we have used this new tool to investigate the role of soluble guanylyl cyclase in the signal transduction of NO in guinea pig trachea and human bronchus.

Materials and Methods

Guinea pig trachea. Male Dunkin-Hartley guinea pigs (250-600 g) (Harlan, Indianapolis, IN) were killed by CO₂ inhalation and exsanguinated, and the trachea were removed. After the removal of fat and connective tissue, the trachea were opened longitudinally opposite the trachealis, and transverse strips consisting of two adjacent cartilage rings were prepared and suspended between platinum ring electrodes in 10-ml organ baths containing a modified Krebs' solution (composition, mM: NaCl 118, CaCl₂ 1.9, KH₂PO₄ 1.0, MgSO₄ 1.2, NaHCO₃ 25.0, glucose 11.1) that contained indomethacin (3 μ M). The Krebs' solution was maintained at 37 °C and gassed with 5% CO₂ in O₂. Tissues were suspended at an initial resting tension of 1.5 g. In previous studies, this tension has been found to be optimal for measuring changes in isometric tension.

After the equilibration period, propranolol (1 μ M) and atropine (1 μ M) were added to inhibit *beta* adrenergic and cholinergic responses,

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ABBREVIATIONS: ODQ, 1H-[1,2,4]oxadiazolol[4,3-a]quinoxalin-1-one; NO, nitric oxide; NANC, nonadrenergic, noncholinergic; SIN-1, 3-morpholinosyndnonimine-N-ethylcarbamide; cGMP, guanosine-3',5'-cyclic monophosphate; 8Br-cGMP, 8-bromoguanosine-3',5'-cyclic monophosphate; VIP, vasoactive intestinal polypeptide; DMSO, dimethyl sulfoxide.

respectively. Then α -chymotrypsin (2 U/ml) was added to inhibit the VIP component of the NANC relaxant response. Tissues were then contracted with 3 μ M histamine to allow relaxant responses to field stimulation to be seen. Once the contraction to histamine had reached a plateau, electrical field current was passed across the tissues. Electrical current was delivered to the electrodes from a Grass SD9 stimulator (Quincy, MA) whose output was passed through a Med-Lab Stimu-Splitter (Fort Collins, CO) for signal monitoring and amplification. The tracheal preparations were stimulated at 8 V, 1 ms, 1 to 16 Hz for 15 s. These stimulation parameters delivered approximately 200 to 400 mA of current across the electrode. Responses to field stimulation were examined in the absence and in the presence of ODQ (0.1, 1 μ M) added 20 min before the first stimulation. Control tissues received the appropriate volume of DMSO, the vehicle for ODQ. Preliminary experiments indicated that DMSO had no effect on responses to field stimulation or to SIN-1. After the final stimulation, the tissues were maximally relaxed with papaverine (100 μ M).

For the experiments with SIN-1, 8Br-cGMP and isoproterenol, tissues were pretreated with atropine (1 μ M) and indomethacin (3 μ M) and contracted with histamine (3 μ M). Concentration response curves to SIN-1 (0.3–100 μ M) were examined in the absence and in the presence of ODQ (0.1, 1 μ M) added 20 min before the start of the curve. Concentration-response curves to 8Br-cGMP (1–100 μ M) and isoproterenol (3–300 nM) were also examined in the absence and in the presence of ODQ (1 μ M) added 20 min before the start of the curve. After the response to the final concentration of relaxant agonist had reached a plateau, 100 μ M papaverine was added to relax the tissues maximally.

Human bronchus. Macroscopically normal human lung tissue was obtained from four patients undergoing surgical resection (Johns Hopkins Hospital, Baltimore, MD) and four organ donors (organ donor tissue was supplied by the Anatomic Gift Foundation, Atlanta, GA). The surgical specimens were from patients with lung carcinoma. Surgical specimens were placed in RPMI 1640 solution (Gibco, Grand Island, NY) at 4°C within 90 min of resection for the 15-min transfer to the laboratory. The organ donor specimens were placed in RPMI 1640 at 4°C and transferred overnight to the laboratory. Upon arrival at the laboratory, the specimens were placed in 4 liters of Krebs' solution gassed with 95% oxygen and 5% carbon dioxide at 4°C. Bronchi (5–10 mm I.D.) were trimmed of surrounding parenchyma and blood vessels. The bronchi were cut longitudinally and prepared as transverse strips 4 to 5 mm wide. Preparations were suspended at an initial tension of 2 g and were washed with fresh buffer every 15 min for a 60-min equilibration period. Experiments similar to those described for guinea pig trachea using SIN-1, 8Br-cGMP and isoproterenol were then undertaken in the human bronchi in the absence and in the presence of ODQ (0.1–10 μ M).

Data analysis. All relaxations were calculated as a percentage of the maximum relaxation induced by papaverine (100 μ M). Statistical analyses were performed using an analysis of variance for repeated measures followed by Student's *t* test for paired observations, where appropriate. P values of less than .05 were considered significant.

Drugs. The following drugs and chemicals were used in this study: atropine sulfate, α -chymotrypsin, histamine phosphate, indomethacin, isoproterenol bitartrate and propranolol (Sigma Chemical Co., St. Louis, MO), ODQ (Tocris Cookson, St. Louis, MO) and 8Br-cGMP (Calbiochem, San Diego, CA). All drugs except indomethacin (ethanol) and ODQ (DMSO) were dissolved in distilled water.

Results

Guinea pig trachea. Field stimulation (1–16 Hz, 8 V, 1 ms for 15 s) of tissues treated with atropine (1 μ M), propranolol (1 μ M), indomethacin (3 μ M), or α -chymotrypsin (2 U/ml) produced rapid, short-lasting relaxations. We have shown

previously that under these conditions, these relaxations are predominantly due to the activation of NO synthase (Ellis and Conanan, 1994; 1995). Relaxations elicited by 2 to 16-Hz field stimulation were partially inhibited by 0.1 μ M ODQ and were substantially inhibited by 1 μ M ODQ (fig. 1).

ODQ (0.1 and 1 μ M) was also found to inhibit relaxations elicited by the NO donor SIN-1 (fig. 2), and 1 μ M produced an almost complete inhibition of relaxations to SIN-1. By contrast, 1 μ M ODQ was without effect on relaxations elicited by the soluble cGMP analog 8Br-cGMP (fig. 3A) or by isoproterenol (fig. 3B). The contractions elicited by histamine (3 μ M) were not altered by either 0.1 or 1 μ M ODQ. Contractions to histamine in the absence of ODQ averaged 1.37 ± 0.12 g ($n = 11$), whereas those in the presence of 0.1 μ M ODQ averaged 1.46 ± 0.15 g ($n = 8$) and those in the presence of 1 μ M ODQ averaged 1.32 ± 0.13 g ($n = 11$).

Human bronchus. ODQ (1 and 10 μ M) was found significantly to inhibit SIN-1-induced relaxations, and an almost complete inhibition of responses occurred with 10 μ M ODQ (fig. 4). In contrast, 10 μ M ODQ was found to be without effect on responses to either 8Br-cGMP (fig. 5A) or isoproterenol (fig. 5B). The contractions elicited by histamine (3 μ M) were not altered by 0.1, 1 μ M or 10 μ M ODQ. Contractions to histamine in the absence of ODQ averaged 1.33 ± 0.27 g ($n = 10$), whereas those in the presence of 0.1 μ M ODQ averaged 1.20 ± 0.28 g ($n = 5$), those in the presence of 1 μ M ODQ averaged 1.18 ± 0.20 g ($n = 5$) and those in the presence of 10 μ M ODQ averaged 1.19 ± 0.26 g ($n = 8$).

Discussion

Our current understanding of the mechanisms by which NO leads to smooth muscle relaxation stems largely from studies with vascular smooth muscle. Numerous studies with vascular smooth muscle support the hypothesis that stimulation of soluble guanylyl cyclase is the initial event in NO-induced relaxation. The role of soluble guanylyl cyclase in responses to NO and other vasodilators in the airways is controversial, however. Confounding the situation is the possibility that there may be species differences in the mechanism of NO on airway smooth muscle function. For example, the relaxations elicited by the NO donor SIN-1 in canine

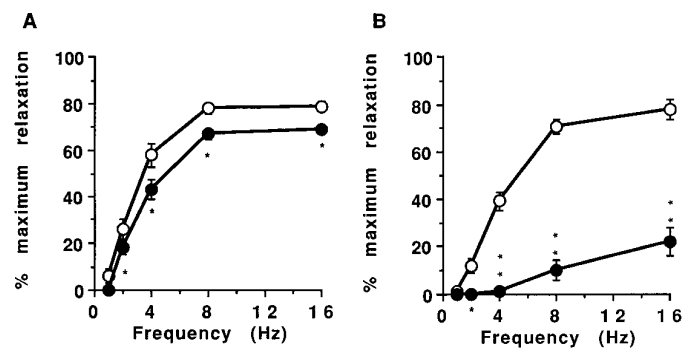


Fig. 1. Ability of the soluble guanylyl cyclase inhibitor ODQ to inhibit relaxations elicited by field stimulation (1–16 Hz, 1 ms, 8 V for 15 s) in guinea pig trachea. Tissues were treated with atropine (1 μ M), propranolol (1 μ M), indomethacin (3 μ M) and α -chymotrypsin (2 U/ml) and contracted with histamine (3 μ M). Results are shown in the absence (○) and in the presence (●) of either 0.1 μ M ODQ (panel A) or 1 μ M ODQ (panel B) and are given as the mean \pm S.E.M., $n = 5$ to 6. * $P < .05$, ** $P < .01$, statistically significant difference in control vs. treated tissues, Student's *t* test for paired results.

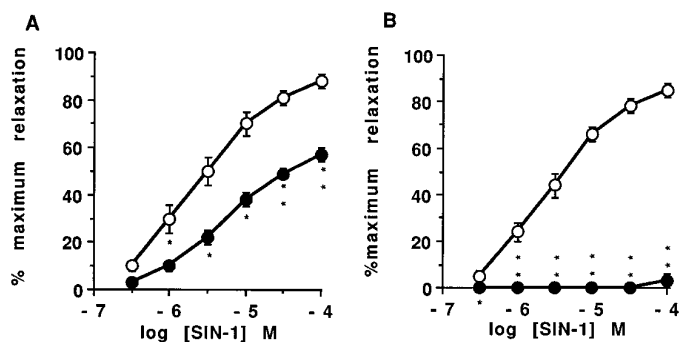


Fig. 2. Effect of the soluble guanylyl cyclase inhibitor ODQ on relaxations to SIN-1 (0.1 μ M ODQ; panel A) and SIN-1 (1 μ M ODQ; panel B) in guinea pig trachea. Tissues were treated with atropine (1 μ M) and indomethacin (3 μ M) and contracted with histamine (3 μ M). Results are shown in the absence (○) and in the presence (●) of ODQ and are given as the mean \pm S.E.M., $n = 5$. * $P < .05$, ** $P < .01$, statistically significant difference in control vs. treated tissues, Student's t test for paired results.

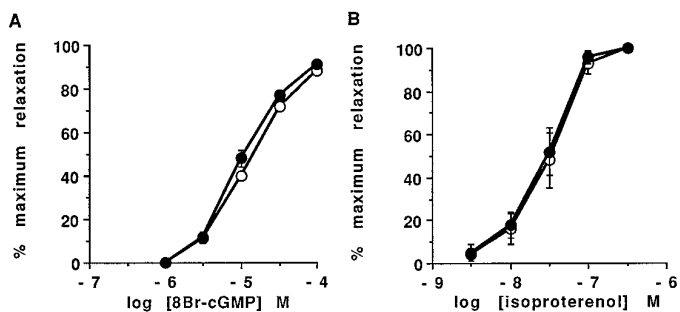


Fig. 3. Effect of the soluble guanylyl cyclase inhibitor ODQ (1 μ M) on relaxations to 8Br-cGMP (panel A) isoproterenol (panel B) in guinea pig trachea. Tissues were treated with atropine (1 μ M) and indomethacin (3 μ M) and contracted with histamine (3 μ M). Results are shown in the absence (○) and in the presence (●) of ODQ and are given as the mean \pm S.E.M., $n = 5$.

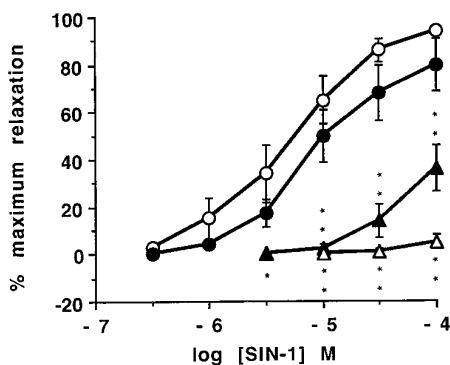


Fig. 4. Effect of the soluble guanylyl cyclase inhibitor ODQ on relaxations to SIN-1 in human bronchus. Tissues were contracted with histamine (3 μ M). Results are shown in the absence (○) and in the presence 0.1 μ M ODQ (●), 1 μ M ODQ (▲) or 10 μ M ODQ (△) and are given as the mean \pm S.E.M., $n = 5$. * $P < .05$, ** $P < .01$, statistically significant difference in control vs. treated tissues, Student's t test for paired results.

trachea (Jones *et al.*, 1994) are inhibited by the soluble guanylyl cyclase inhibitor methylene blue, whereas relaxations in porcine airways are not (Stuart-Smith *et al.*, 1994). Similar species differences have been obtained with sodium nitroprusside (SNP) (Zhou and Torphy, 1991; Hamaguchi *et al.*, 1992; Stuart-Smith *et al.*, 1994). Even though the relaxations

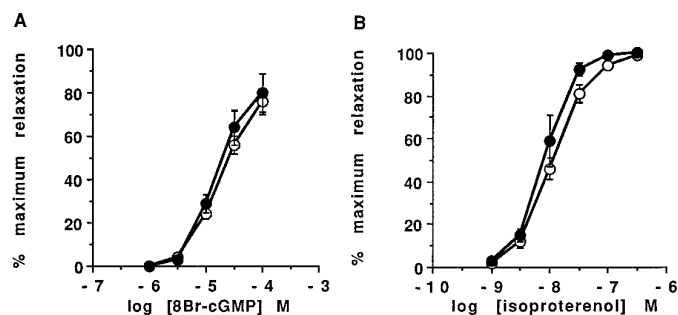


Fig. 5. Effect of the soluble guanylyl cyclase inhibitor ODQ (10 μ M) on relaxations to 8Br-cGMP (panel A) and isoproterenol (panel B) in human bronchus. Tissues were contracted with histamine (3 μ M). Results are shown in the absence (○) and in the presence (●) of ODQ and are given as the mean \pm S.E.M., $n = 5$.

to SNP were not inhibited by methylene blue in the study by Zhou and Torphy (1991), the elevation of cGMP by SNP was inhibited by this agent, which suggests that cGMP is not involved in this response. Recent studies in guinea pig airways have also suggested a dichotomy between relaxations to NO and accumulation of cGMP (Lindsay *et al.*, 1995; Mahey *et al.*, 1995).

Studies in human airways have also yielded conflicting results. Thus electrical field stimulation of human tracheal tissue, under conditions that selectively elicit relaxations that are blocked by NO synthase inhibitors, increased the levels of cGMP. The increase of cGMP, as well as the relaxation of the trachealis, was inhibited by methylene blue (Ward *et al.*, 1995). Gaston *et al.*, (1994) also noted that NO donors and NO itself increased cGMP levels in human airways. They found, however, that although methylene blue blocked the NO-induced cGMP elevations, it had no effect on the relaxations, which again suggests that pathways other than, or in addition to, cGMP are involved in the NO response.

A significant limitation in all of these studies was the lack of powerful pharmacological tools that could be used to study the soluble guanylyl cyclase system. Nearly all of the studies relied on methylene blue to inhibit soluble guanylyl cyclase, but this compound is a very weak inhibitor of soluble guanylyl cyclase and, moreover, possesses other activities, including generation of superoxide anions (Wolin *et al.*, 1991; Marczin *et al.*, 1992) and inhibition of NO synthase (Mayer *et al.*, 1993). Recently, however, a very potent and selective inhibitor of soluble guanylyl cyclase has been identified (Garthwaite *et al.*, 1995). The compound identified in this study, ODQ, has nanomolar sensitivity against soluble guanylyl cyclase (Garthwaite *et al.*, 1995). ODQ appears to be very selective for soluble guanylyl cyclase in that it was ineffective at concentrations up to 100 μ M against particulate guanylyl cyclase or adenylyl cyclase (Garthwaite *et al.*, 1995). Nor does this compound inhibit NO synthase or produce superoxide anions (Garthwaite *et al.*, 1995). These reports on the selectivity of ODQ for soluble guanylyl cyclase have recently been confirmed (Moro *et al.*, 1996). This study found that ODQ did not inhibit particulate guanylyl cyclase or NO synthase and moreover did not interfere with the ability of an NO donor to release NO (Moro *et al.*, 1996).

Relaxations elicited by the NO component of the NANC relaxant system and by the NO donor SIN-1 were substantially inhibited by the soluble guanylyl cyclase inhibitor ODQ

(Garthwaite *et al.*, 1995). These results provide strong evidence for a role for this enzyme in the signal transduction of NO responses in guinea pig trachea and human bronchus. ODQ is a recently reported inhibitor of NO-sensitive guanylyl cyclase, and appropriate care should be taken when interpreting results of the use of a novel compound such as ODQ. This notwithstanding, in the original paper describing this compound, several key controls were carried out to test its selectivity (Garthwaite *et al.*, 1995). ODQ at concentrations higher than those used in the present study did not inhibit neuronal NO synthase, did not directly inactivate NO and did not produce superoxide anions. Furthermore, ODQ is selective for soluble guanylyl cyclase, being without effect on particulate guanylyl cyclase or on adenylyl cyclase (Garthwaite *et al.*, 1995). Further controls to test the selectivity of ODQ were carried out in the present study. ODQ had no effect on relaxations to the cGMP analog 8Br-cGMP, which acts downstream of the activation of soluble guanylyl cyclase. ODQ was also without effect on relaxations elicited by isoproterenol, which acts primarily *via* the activation of adenylyl cyclase (Lohmann *et al.*, 1977).

We observed that a greater concentration of ODQ was needed almost to abolish SIN-1-induced relaxations in human bronchus than in guinea pig trachea. This may be due to a number of factors, including 1) different penetration of ODQ between the tissue types, 2) greater stimulation of soluble guanylyl cyclase by SIN-1 in human than in guinea pig tissue, 3) differential sensitivity of the muscles to cGMP and 4) differences in phosphodiesterase activity. ODQ may also be a less potent inhibitor of human soluble guanylyl cyclase than of guinea pig soluble guanylyl cyclase. Which of these factors are important remains to be determined.

In conclusion, these results strongly support a role for the activation of soluble guanylyl cyclase in responses to NO in guinea pig trachea and human bronchus. Furthermore, it is likely that ODQ will be a useful tool in elucidating the role of NO activation of soluble guanylyl cyclase in many organ systems.

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