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The role of cGMP in the relaxation to nitric oxide donors in airway smooth muscle

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Abstract

The aim of this study was to determine the effect of the soluble guanylyl cyclase inhibitors methylene blue and LY83583 (6-anilino-5,8-quinolinedione) on relaxation and increases in intracellular guanosine 3′,5′-cyclic monophosphate (cGMP) concentration ($[cGMP]_i$) induced by sodium nitroprusside, 3-morpholinosydnonimine (SIN-1) and diethylamine–nitric oxide (NO) in porcine tracheal smooth muscle in vitro. We measured (1) the effect of NO donors on isometric force and $[cGMP]_i$ and (2) the ability of methylene blue and LY83583 to antagonize these effects. In muscle strips contracted with carbachol (0.1–0.3 μM), both sodium nitroprusside and diethylamine–NO caused relaxation and an increase in $[cGMP]_i$. By contrast, SIN-1 caused a relaxation which was not associated with a concomitant increase in $[cGMP]_i$. Methylene blue (10 μM) and LY83583 (10 μM) completely blocked the increase in $[cGMP]_i$ induced by sodium nitroprusside and diethylamine–NO; however substantial relaxation remained. It is concluded that in porcine airway smooth muscle, (1) relaxation induced by some NO donors may occur without a concomitant increase in $[cGMP]_i$; and (2) whereas relaxation induced by some NO donors may be associated with increases in $[cGMP]_i$, the relaxation is not completely dependent upon it. © 1998 Elsevier Science B.V.

Keywords: Smooth muscle, airway; cGMP; LY83583; Methylene blue; Nitric oxide (NO); Sodium nitroprusside; 3-Morpholinosydnonimine; Diethylamine–nitric oxide

1. Introduction

Nitric oxide (NO) plays an important physiologic role in several biological systems, including the systemic and pulmonary vasculature (Johns, 1991). Clinically important compounds that generate NO (i.e., NO donors), such as sodium nitroprusside and 3-morpholinosydnonimine (SIN-1), are thought to act by releasing NO (Murad et al., 1978; Bates et al., 1991; Feelisch, 1991; Johns, 1991). Although their mechanism of action is not fully known, it is believed that these compounds relax vascular smooth muscle via activation of soluble guanylyl cyclase and an increase in intracellular guanosine 3′,5′-cyclic monophosphate (cGMP) concentration ($[cGMP]_i$) (Kukovetz et al., 1979; Koesling et al., 1991).

NO and NO donors also relax airway smooth muscle in vitro (Zhou and Torphy, 1991; Gaston et al., 1994; Jones et al., 1994; Stuart-Smith et al., 1994). However, in this tissue, the role of cGMP in mediating this relaxation is not clear. In canine tracheal smooth muscle, both relaxation and the increase in $[cGMP]_i$ induced by *S*-nitroso-*N*-acetyl penicillamine (Zhou and Torphy, 1991) or SIN-1 (Jones et al., 1994) are inhibited by the putative soluble guanylyl cyclase inhibitor methylene blue. In contrast, relaxation of human bronchi induced by *S*-nitroso-*N*-acetyl-cysteine is unaffected by methylene blue, although increases in $[cGMP]_i$ are attenuated (Gaston et al., 1994). In porcine airway smooth muscle, relaxation induced by sodium nitroprusside and SIN-1 is not altered by methylene blue, although the relaxation induced by NO is inhibited (Stuart-Smith et al., 1994); $[cGMP]_i$ was not measured in that study. Taken together, these data suggest that the relaxation produced by some NO donors may not involve

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soluble guanylyl cyclase and cGMP, although NO may relax airway smooth muscle primarily via cGMP.

Since putative NO donors are thought to relax smooth muscle by releasing NO, the question arises as to why some NO donors behave differently with regard to cGMP as compared to NO. We hypothesized that some putative NO donors relax airway smooth muscle in part independently of activation of soluble guanylyl cyclase and an increase in $[cGMP]_i$. To test this hypothesis, we examined the relaxation response of porcine tracheal smooth muscle in vitro to various putative NO donors in the presence and absence of the soluble guanylyl cyclase inhibitors methylene blue and 6-anilino-5,8-quinolinedione (LY83583). In addition, the effect of these inhibitors on increases in $[cGMP]_i$ induced by these agents was examined to determine whether the amount of relaxation was correlated with increases in $[cGMP]_i$. Two clinically relevant NO donors, sodium nitroprusside and SIN-1, were chosen for this study. Their effects on relaxation and $[cGMP]_i$ were compared with that induced by the NO-nucleophile adduct diethylamine–NO, which releases NO spontaneously in aqueous solution (Maragos et al., 1991, Morley et al., 1993).

2. Methods

2.1. Techniques

2.1.1. Tissue preparation

Domestic pigs (female, weight 30–50 kg) were sacrificed by injection of a lethal dose of sodium pentobarbital (250 mg bolus followed by 25 mg/kg intravenously). The tracheas were excised and placed in cold physiological salt solution (PSS) of the following composition (mM): 110.5 NaCl, 25.7 $NaHCO_3$, 5.6 dextrose, 3.4 KCl, 2.4 $CaCl_2$, 1.2 KH_2PO_4 and 0.8 $MgSO_4$. Tissues were dissected free of cartilage and connective tissue and the epithelium was removed. The tracheal muscle was then cut into strips (approximately 1.5 cm long and 3 mm wide) which were suspended in 25 ml water-jacketed tissue baths containing:, PSS (37°C, 94% O_2/6% CO_2, pH 7.4). One end of the strips was secured to the bottom of the tissue bath via a metal hook; the other end was attached to a calibrated force transducer (Grass Instruments, Quincy, MA, model FT031).

Following 30 min of equilibration at zero tension, the strips were contracted isometrically for 30 seconds every 5 min by supramaximal electrical field stimulation (400 mA, 25 Hz, 0.5 ms puIse duration). Electrical field stimulation was triggered by a stimulator (Grass Instruments, model S88D) and delivered by a direct current amplifier (Section of Engineering, Mayo Foundation). The length of the strips was increased after each stimulation until active isometric force was maximal (resting tension at optimal length approximately 0.4–1.4 g). The tissues were maintained at this optimal length for the remainder of the experiment.

2.1.2. Measurement of $[cGMP]_i$

Frozen muscle strips were weighed and homogenized in 4 ml of cold (2°C) 95% ethanol by using a ground–glass pestle and homogenizing tube. The precipitated pellet was separated from the soluble extract by centrifugation at 4,000 *g* for 10 min. The soluble extract was evaporated to dryness at ~55°C under a stream of nitrogen and was then suspended in 0.5 ml of 4 mM EDTA (pH 7.5). [^{3}H]cGMP (0.4 μCi) was added as a tracer for recovery determinations. Commercially available radioimmunoassay kits were used to determine $[cGMP]_i$ in the soluble extract (Brooker et al., 1979). The protein content of the precipitated pellet was determined by the method described by Lowry et al. (1951) using bovine serum albumin dissolved in 1 N NaOH as the standard. $[cGMP]_i$ was expressed as picomoles per milligram protein.

2.2. Experimental protocols

In airway smooth muscle, the degree of relaxation induced by various compounds depends on the initial degree of active isometric force (i.e., initial isometric force). Higher levels of contraction reduce both maximal relaxation and the sensitivity of the tissue to relaxing agents, a phenomenon referred to as functional antagonism (Torphy et al., 1985). To eliminate the effects of functional antagonism, preliminary experiments were performed in which concentration–response curves to carbachol (10^{-9}–10^{-4} M) were generated for each muscle strip in the absence (control), or in the presence of 10 μM methylene blue or 10 μM LY83583. In these and all subsequent experiments, the soluble guanylyl cyclase inhibitors were added to the tissue baths 30 min before the start of the experiment and throughout the concentration–response curve. Neither methylene blue nor LY83583 had any effect on baseline force or the concentration of carbachol that produced an isometric force of 50% of maximal (EC_{50}) (data not shown). All muscle strips were then contracted with the EC_{50} for carbachol. This ensured that the initial isometric forces generated by carbachol were similar for each muscle strip so that variations in initial isometric force did not affect the relaxation responses to the NO donors. All tissues were incubated with 10 μM indomethacin to prevent the production of endogenous prostanoids that could affect cyclic nucleotide levels (Jones et al., 1994).

2.2.1. Effect of methylene blue on relaxation induced by NO donors

For each NO donor, two muscle strips were studied. One strip was incubated with 10 μM methylene blue for 20 min. The second strip was not incubated with methylene blue and was used to provide control responses to the NO donors. The tissues were then contracted with an EC_{50}

for carbachol which was determined as described above. After initial isometric force had stabilized (6 min), concentration–response curves were generated with either sodium nitroprusside (10^{-9}–10^{-4} M), SIN-1 (10^{-9}–10^{-4} M), or diethylamine–NO (10^{-8}–10^{-5} M). This protocol also determined the concentration of the NO donors producing either 25% (EC_{25}), 50% (EC_{50}), or 75% (EC_{75}) of maximal relaxation, which was used for the subsequent protocol. Methylene blue was selected as the soluble guanylyl cyclase inhibitor for this experimental protocol because it has previously been used in this laboratory to demonstrate that NO donor-mediated relaxation with SIN-1 is cGMP contingent in airway smooth muscle. In preliminary studies, concentrations of methylene blue >10 μM caused spontaneous contractions.

2.3. Effect of guanylyl cyclase inhibitors on isometric force and $[cGMP]_i$

For each NO donor, four muscle strips were contracted with an EC_{50} concentration of carbachol until isometric force was stable (6 min). One strip was not exposed to an NO donor and was rapidly frozen by immersion in liquid nitrogen for 30 s to obtain the baseline $[cGMP]_i$. The other three strips were exposed to a concentration of an NO donor that produced 25%, 50%, or 75% of the maximal relaxation for that drug (determined from the previous protocol). The concentrations of the NO donors used were 0.1, 0.3, and 1 μM for sodium nitroprusside, 0.3, 1.0 and 3.0 μM for SIN-1, and 0.03, 0.1 and 0.3 μM for diethylamine–NO. The time to maximal relaxation was different for each drug, 2 min for sodium nitroprusside, 4 min for SIN-1, and 2 min for diethylamine–NO. The strips were rapidly immersed in liquid nitrogen for 30 s at maximal relaxation. The strips were kept frozen at -70°C until $[cGMP]_i$ measurements were made. This protocol was then repeated in strips incubated with 10 μM methylene blue or 10 μM LY83583.

2.4. Drugs

SIN-1 was a gift from Dr. Henning, Cassella–Riedel, Germany. diethylamine–NO was purchased from Cayman Chemical, Ann Arbor, MI and LY83583 was purchased from Research Biochemicals, Natick. MA. All other chemicals were purchased from Sigma Chemical, St. Louis, MO. Diethylamine–NO was prepared in Tris buffer at pH 8.8; all other drugs were dissolved in distilled water. Radioimmunoassay kits for cGMP were purchased from Amersham, Arlington Heights, IL. Reagents for protein determinations were purchased from Bio-Rad Laboratories, Hercules, CA.

2.5. Statistical analysis

Data are expressed as means values ± standard error of the mean (S.E.M.); n represents the number of pigs studied. Concentration–response curves were compared by nonlinear regression analysis as described by Meddings et al. (1989). All other data were compared by repeated measures analysis of variance (ANOVA) with post hoc analysis by Student's *t*-test with Bonferroni correction for multiple comparison. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of methylene blue on relaxation induced by NO donors

There was no significant difference in the EC_{50} for carbachol (0.1–0.3 μM) between strips subsequently relaxed by sodium nitroprusside, SIN-1, or diethylamine–NO. Preliminary studies indicated that the parent compound for diethylamine–NO (diethylamine, 0.01–100 μM) and the only other metabolite of diethylamine–NO, *N*-nitrosodiethylamine (0.1–100 μM) had no effect on isometric force induced by carbachol (data not shown).

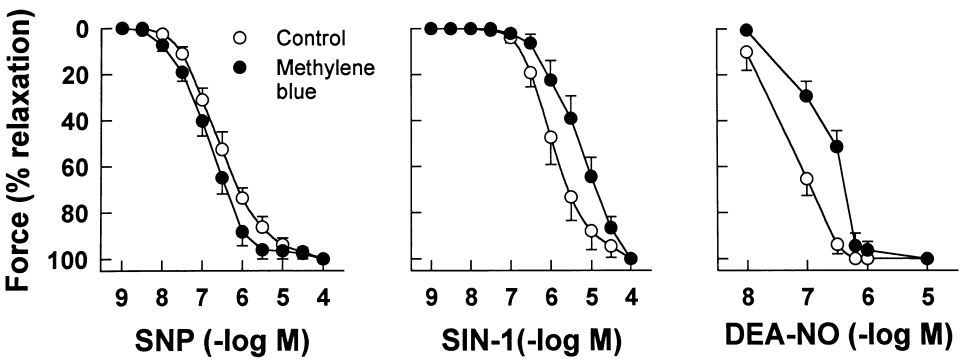

Fig. 1. Effect of 10 μM methylene blue on relaxation induced by sodium nitroprusside (SNP, left panel), 3-morpholinosydnonimine (SIN-1, middle panel), and diethylamine–NO (DEA–NO, right panel) in porcine tracheal smooth muscle contracted with 0.1–0.3 μM carbachol. Data are mean ± S.E.M; $n = 7$.

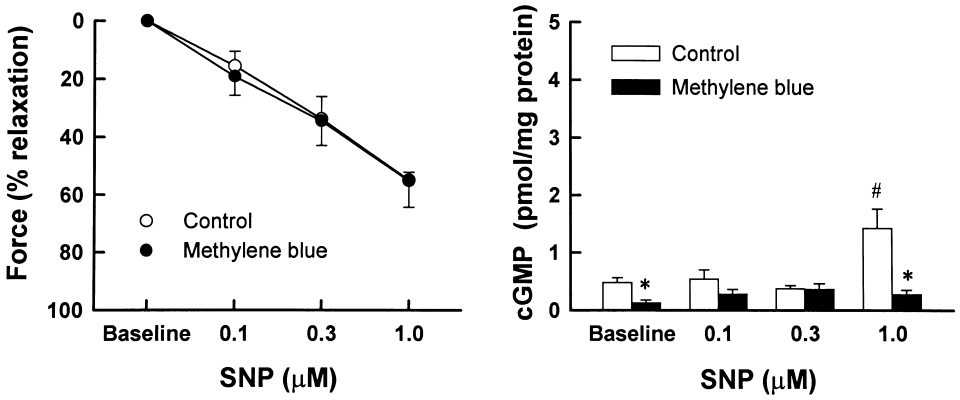

Fig. 2. Effect of sodium nitroprusside on isometric force (left panel) and guanosine ′3,5′-cyclic monophosphate concentration ($[cGMP]_i$, right panel) in porcine tracheal smooth muscle contracted with 0.1–0.3 μM carbachol in the absence (control) and presence of 10 μM methylene blue. * Significantly different from control values. # Significantly different from baseline $[cGMP]_i$. Data are the mean ± S.E.M.; $n = 6$.

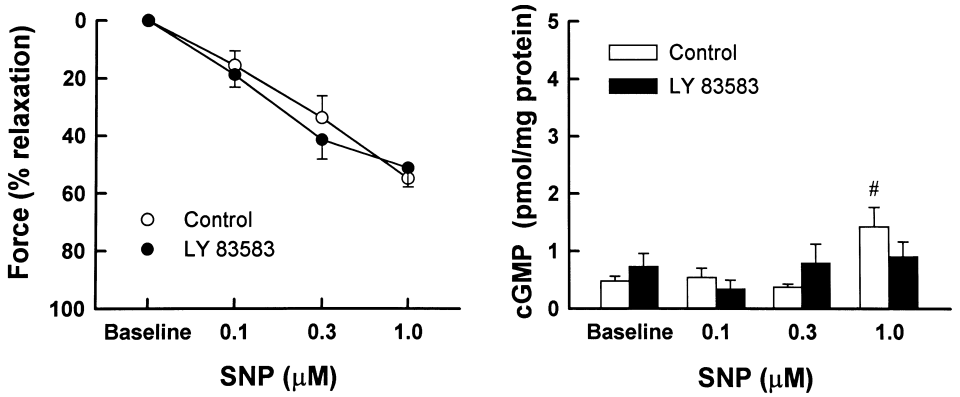

Fig. 3. Effect of sodium nitroprusside on isometric force (left panel) and guanosine 3′,5′-cyclic monophosphate concentration ($[cGMP]_i$, right panel) in porcine tracheal smooth muscle contracted with 0.1–0.3 μM carbachol in the absence (control) and presence of 10 μM LY83583. * Significantly different from control values. # Significantly different from baseline $[cGMP]_i$. Data are the mean ± S.E.M.; $n = 6$.

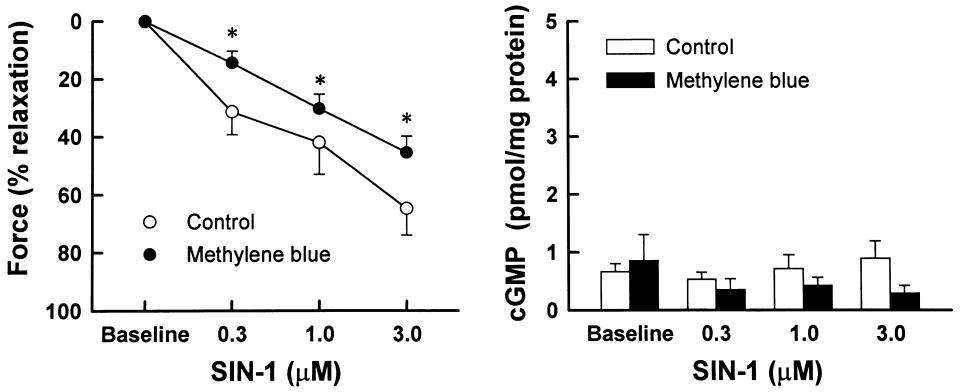

Fig. 4. Effect of 3-morpholinosydnonimine (SIN-1) on isometric force (left panel) and guanosine 3′,5′-cyclic monophosphate concentration ($[cGMP]_i$, right panel) in porcine tracheal smooth muscle contracted with 0.1–0.3 μM carbachol in the absence (control) and presence of 10 μM methylene blue. * Significantly different from control values. # Significantly different from baseline $[cGMP]_i$. Data are the mean ± S.E.M.; $n = 6$.

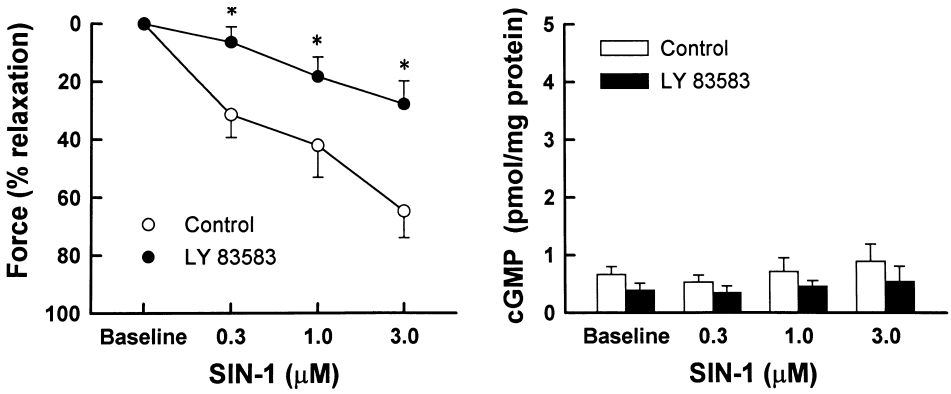

Fig. 5. Effect of 3-morpholinosydnonimine (SIN-1) on isometric force (left panel) and guanosine 3′,5′-cyclic monophosphate concentration ($[cGMP]_i$, right panel) in porcine tracheal smooth muscle contracted with 0.1–0.3 μM carbachol in the absence (control) and presence of 10 μM LY83585. * Significantly different from control values. # Significantly different from baseline $[cGMP]_i$. Data are the mean ± S.E.M.; $n = 6$.

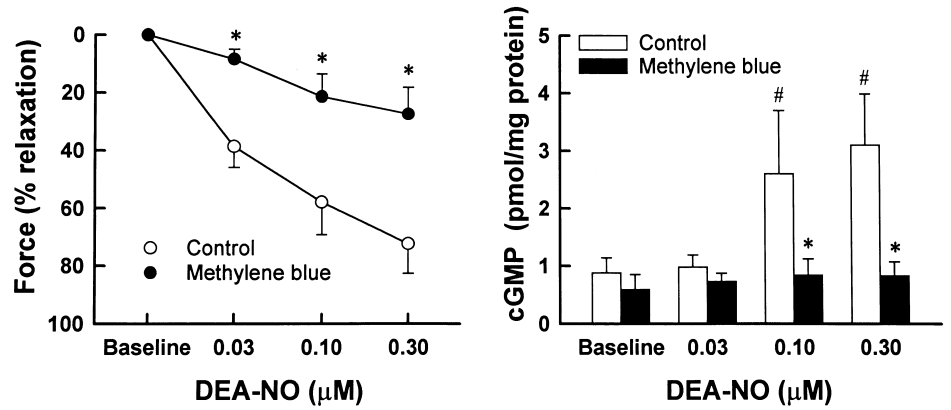

Fig. 6. Effect of diethylamine–NO on isometric force (left panel) and guanosine 3′,5′-cyclic monophosphate concentration ($[cGMP]_i$, right panel) in porcine tracheal smooth muscle contracted with 0.1–0.3 μM carbachol in the absence (control) and presence of 10 μM methylene blue. * Significantly different from control values. # Significantly different from baseline $[cGMP]_i$. Data are the mean ± S.E.M.; $n = 7$.

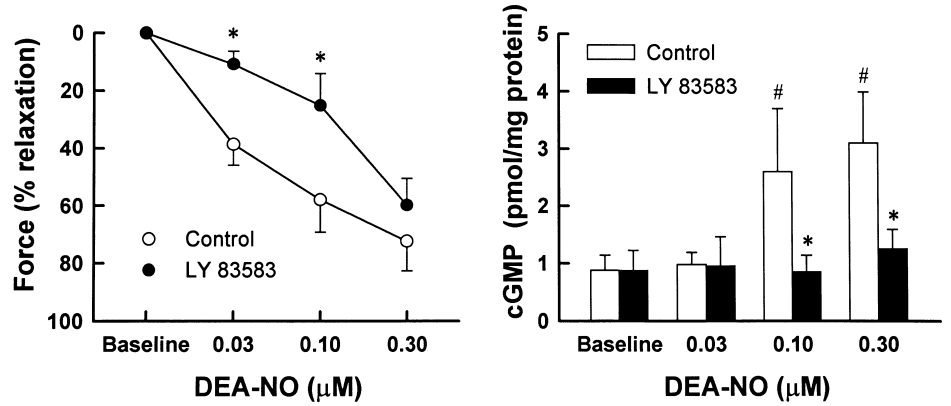

Fig. 7. Effect of diethylamine-NO on isometric force (left panel) and guanosine 3′,5′-cyclic monophosphate concentration ($[cGMP]_i$, right panel) in porcine tracheal smooth muscle contracted with 0.1–0.3 μM carbachol in the absence (control) and presence of 10 μM LY83583. * Significantly different from control values. # Significantly different from baseline $[cGMP]_i$. Data are the mean ± S.E.M.; $n = 7$.

After contractions induced by carbachol had stabilized, cumulative addition of sodium nitroprusside (Fig. 1, left panel), SIN-1 (Fig. 1, middle panel) and diethylamine–NO (Fig. 1, right panel) to the tissue baths caused a concentration-dependent decrease in isometric force. The EC_{50} values were 0.43 ± 0.06 μM, 1.2 ± 0.2 μM, and 0.06 ± 0.01 μM for sodium nitroprusside, SIN-1, and diethylamine–NO respectively. Methylene blue significantly increased the EC_{50} for SIN-1 (from 1.2 ± 0.02 μM to 5.0 ± 0.9 μM) and diethylamine–NO (from 0.06 ± 0.01 μM to 0.24 ± 0.05 μM). In contrast, methylene blue significantly decreased the EC_{50} for sodium nitroprusside (from 0.43 ± 0.06 μM to 0.14 ± 0.02 μM). Thus, methylene blue significantly attenuated relaxation induced by SIN-1 and diethylamine–NO, and significantly augmented relaxation induced by sodium nitroprusside.

3.2. Effect of guanylyl cyclase inhibitors on isometric force and $[cGMP]_i$

After contractions induced by carbachol had stabilized, non-cumulative addition of sodium nitroprusside (0.1, 0.3 or 1 μM) caused a concentration-dependent decrease in isometric force (Figs. 2 and 3, left panel). Additionally, sodium nitroprusside caused a significant increase in $[cGMP]_i$, but only at 1 μM (Fig. 2, right panel). Neither methylene blue (Fig. 2, left panel) nor LY83583 (Fig. 3, left panel) altered the relaxation induced by sodium nitroprusside. However, methylene blue completely abolished the increase in $[cGMP]_i$, (Fig. 2, right panel). Although LY83583 partially attenuated the increase in $[cGMPl_i$ induced by sodium nitroprusside, the effect was not statistically significant (Fig. 3, right panel; $P = 0.06$). It is of interest to note that methylene blue decreased basal $[cGMP]_i$, a finding that previously has not been demonstrated for airway smooth muscle and most likely is a random finding contained within the variability of the low basal $[cGMP]_i$.

As with sodium nitroprusside, SIN-1 (0.3, 1.0 and 3.0 μM) caused a concentration-dependent decrease in isometric force (Figs. 4 and 5, left panel). However, $[cGMP]_i$ was not increase by SIN-1 (Figs. 4 and 5, right panel). Both methylene blue and LY83583 significantly decreased in the sensitivity of the strips to SIN-1 (Figs. 4 and 5, left panel), but had no effect on the already low $[cGMP]_i$ (Figs. 4 and 5, right panel).

Diethylamine–NO (0.03, 0.1, and 0.3 μM) caused a concentration-dependent decrease in isometric force (Figs. 6 and 7, left panel) and a significant increase in $[cGMP]_i$ at 0.1 and 0.3 μM; the relaxation induced by 0.03 μM diethylamine–NO was not accompanied by an increase $[cGMP]_i$ (Figs. 6 and 7, right panel). Both methylene blue and LY83583 significantly decreased the sensitivity of the strips to diethylamine–NO (Figs. 6 and 7, left panel). However, for LY83583, there was no significant difference in the amount of relaxation induced by 0.3 μM diethylamine–NO. Both methylene blue and LY83583 abolished the increase in $[cGMP]_i$ induced by 0.1 and 0.3 μM diethylamine–NO (Figs. 6 and 7, right panel). However, a significant proportion of the relaxation remained (Figs. 6 and 7, left panel).

4. Discussion

These results demonstrate that in porcine tracheal smooth muscle contracted with carbachol: (1) some NO donors can induce considerable relaxation without a concomitant increase in $[cGMP]_i$; (2) whereas relaxation induced by some NO donors may be associated with increases in $[cGMP]_i$, the relaxation is not completely dependent upon it; and (3) methylene blue and LY83583 may attenuate relaxation induced by some NO donors independently of inhibitory effects on soluble guanylyl cyclase.

The role of cGMP in mediating relaxation induced by NO donors is not clear. Work by Zhou and Torphy (1991) showed that in canine tracheal smooth muscle, relaxation induced by sodium nitroprusside was actually augmented by methylene blue, even though the sodium nitroprusside-induced increase in $[cGMP]_i$ was inhibited. In guinea-pig tracheal smooth muscle relaxation induced by *S*-nitrosothiols is only partially mediated by an increase in $[cGMP]_i$ (Jansen et al., 1992). Lastly, we previously demonstrated the inability of methylene blue to inhibit relaxation induced by nitrovasodilators (Stuart-Smith et al., 1994). The present paper confirms these observations, and extends them to a second inhibitor of guanylyl cyclase, LY83583 (Schmidt et al., 1985). Furthermore, the results presented in this study demonstrate a clear dissociation between an increase in $[cGMP]_i$ and relaxation of porcine airway smooth muscle. Each of the nitrovasodilators examined have responded differently in the presence of the soluble guanylyl cyclase inhibitors, indicating different mechanisms of action of the so-called nitrovasodilators.

In the present study, sodium nitroprusside caused a concentration-dependent relaxation of the airway smooth muscle. At low concentrations of the agonist, significant relaxation occurred without a concomitant increase in $[cGMP]_i$. At higher concentrations of sodium nitroprusside (1 μM), relaxation was associated with an increase in $[cGMP]_i$. However, whereas both methylene blue and LY83583 inhibited the increase in $[cGMP]_i$ induced by sodium nitroprusside, relaxation was either not significantly affected (Fig. 2) or in some experiments with methylene blue, slightly augmented (Fig. 1), as previously reported for canine tracheal smooth muscle (Zhou and Torphy, 1991) and rabbit aorta (Gryglewski et al., 1992). These results indicate that the relaxation induced by sodium nitroprusside is substantially independent of changes in $[cGMP]_i$.

The mechanism by which methylene blue potentiates relaxation induced by sodium nitroprusside is not known.

In contrast to both the current study that conducted in canine tracheal smooth muscle (Zhou and Torphy, 1991) in which methylene blue blocked the increase in $[cGMP]_i$ induced by sodium nitroprusside, Gryglewski et al. (1992) found that methylene blue actually augmented the increase in $[cGMP]_i$. The mechanism for this increase in $[cGMP]_i$ is currently inexplicable, and we did not see it in our experiments. Alternatively, methylene blue generates superoxide anion, an action that is apparently distinct from its ability to inhibit soluble guanylyl cyclase (Wolin et al., 1990). It has been postulated that the effects of methylene blue on relaxation induced by NO donors may be related to the different amounts of superoxide formation under different experimental conditions (Wolin et al., 1990; Khan et al., 1993). Although, currently there is no direct evidence that superoxide anion could enhance the sensitivity of airway smooth muscle to sodium nitroprusside, previous studies have demonstrated that oxidative stress induced by hydrogen peroxide can relax airway smooth muscle (Gao and Vanhoutte, 1992).

How might this relaxation occur? Sodium nitroprusside is thought to act by releasing NO (Bates et al., 1991; Kowaluk et al., 1992). It is not thought to release NO spontaneously, but seems to require the presence of light or the addition of subcellular fractions of smooth muscle (Bates et al., 1991; Kowaluk et al., 1992). That is, sodium nitroprusside requires intracellular activation before NO is released (Kowaluk et al., 1992). The released NO may act in one of two ways. One route of NO action is via an interaction with the heme moiety of the soluble guanylyl cyclase enzyme (Koesling et al., 1991), causing an increase in $[cGMP]_i$ and hence relaxation. The second is by oxidation of intracellular thiols on proteins that regulate smooth muscle contraction, either by *S*-nitrosylation of protein or other intracellular thiols (Stamler et al., 1992; Lipton et al., 1993), or reversible oxidation of protein thiols without *S*-nitrosylation (Stamler et al., 1992). For example, both NO and sodium nitroprusside cause *S*-nitrosylation of protein kinase C, leading to its inactivation (Gopalakrishna et al., 1993). It is possible that sodium nitroprusside releases NO close to the proteins involved in contraction, leading to *S*-nitrosylation or reversible thiol oxidation, inactivation of the proteins, and relaxation.

SIN-1 caused concentration-dependent relaxations which were not associated with any increase in $[cGMP]_i$. However, both methylene blue and LY83583 inhibited relaxation. This finding may be related to the chemistry of SIN-1. In oxygenated aqueous solutions, SIN-1 is hydrolyzed to an open-ring A form, SIN-1A. SIN-1A then undergoes an autocatalytic process involving molecular oxygen to yield NO and superoxide anion (Feelisch et al., 1989; Augusto et al., 1994). This spontaneous release of NO is thought to account for the biological activity of SIN-1 (Feelisch, 1991). However, the released NO and superoxide can combine to form peroxynitrite (Augusto et al., 1994). Peroxynitrite is known to cause relaxation of vascular smooth muscle (Liu et al., 1994; Wu et al., 1994). It is conceivable that SIN-1 causes relaxation of airway smooth muscle by formation of peroxynitrite. The relaxation to peroxynitrite in pulmonary arteries is partially inhibited by both methylene blue and LY83583 (Wu et al., 1994). However, the present results indicate that this is not due to inhibition of soluble guanylyl cyclase. Both methylene blue and LY83583 are electron acceptors, with redox capabilities (Fukahori et al., 1994). It may be postulated that in the presence of these agents, an oxidation reaction takes place, resulting in the inactivation of peroxynitrite and thus inhibition of the relaxation to SIN-1. The mechanism by which peroxynitrite might cause relaxation that is independent of an increase in $[cGMP]_i$ is unknown. As with NO, peroxynitrite is also able to oxidize reduced thiols such as glutathione (Augusto et al., 1994; Wu et al., 1994). Thus, it is possible that SIN-1 acts via a thiol-dependent pathway in a similar manner postulated for sodium nitroprusside.

Diethylamine–NO is an NO/nucleophile adduct which spontaneously releases NO extracellularly by a non-enzymatic process (Maragos et al., 1991; Maragos et al., 1993; Morley et al., 1993). In aqueous salt solutions, the rate of NO release is dependent only on the pH and temperature of the solution (Maragos et al., 1991; Morley et al., 1993). At pH 7.4 and temperature 37°C, diethylamine–NO generates 1.5 mol of NO per mole of the adduct, with a half-life of 2.1 min (Maragos et al., 1991). Thus, diethylamine–NO generates large quantities of NO very rapidly in a predictable manner, following first order kinetics (Morley et al., 1993). In the current study the estimated peak concentrations of NO achieved in the tissue baths were 0.04, 0.12, and 0.32 μM for 0.03, 0.1 and 0.3 μM diethylamine–NO, respectively (Hirasaki et al., 1996). These concentrations are far lower than those previously obtained using NO gas in aqueous solution (Stuart-Smith et al., 1994). NO gas is known to cause relaxation of airway smooth muscle (Stuart-Smith et al., 1994). However, NO delivered by this method requires high concentrations of NO gas, and the final concentration in the oxygen-containing solution is unpredictable. The present experiments confirm that, under physiological conditions, very small quantities of NO released from diethylamine–NO can cause relaxation of tracheal smooth muscle (Hirasaki et al., 1996). Thus, airway smooth muscle is much more sensitive to NO than previously thought. However, the mechanism by which diethylamine–NO causes relaxation is not clear. Certainly, diethylamine–NO causes concentration-dependent relaxation that in general, is accompanied by a concentration-dependent increase in $[cGMP]_i$ in the porcine tracheal smooth muscle. However, there are two lines of evidence to suggest that the relaxation induced by diethylamine–NO, although associated with an increase in $[cGMP]_i$ is not entirely dependent upon this increase. First, diethylamine–NO can relax porcine tracheal smooth muscle without an associated increase in

$[cGMP]_i$, as $[cGMP]_i$ increases only at the higher concentrations of the agonist; O.03 μM diethylamine–NO caused ~40% relaxation without any detectable increase in $[cGMP]_i$. Second, when the increases in $[cGMP]_i$ were completely blocked by both methylene blue and LY83583, marked relaxation persisted, indicating that the relaxation induced by diethylamine–NO was in part independent of an increase in $[cGMP]_i$.

Although little is known regarding these cGMP-independent mechanisms, the NO generated by diethylamine–NO may enter the smooth muscle cell and oxidize intracellular thiols, as postulated for other NO donors. It has been shown in vascular smooth muscle that NO may induce relaxation via stimulation of calcium-activated potassium channels by a mechanism independent of cGMP (Khan et al., 1993; Bolotina et al., 1994). These channels are known to be present in airway smooth muscle (Garcia-Calvo et al., 1994) and when activated, hyperpolarize the cell membrane and decrease cytosolic Ca^{2+} concentration, thereby causing relaxation. Thus, it is possible that a proportion of the relaxation induced by diethylamine–NO is mediated by activation of calcium-activated potassium channels. This would explain the persistence of the relaxation in the face of complete inhibition of the increase in $[cGMP]_i$.

It also must be noted that both soluble guanylyl cyclase inhibitors attenuated the relaxation induced by low concentrations of diethylamine–NO, which did not increase $[cGMP]_i$. The explanation is most likely similar to that for SIN-1, which primarily induced relaxation without a concomitant increase in $[cGMP]_i$; i.e., that the NO released in aqueous solution was scavenged by the soluble guanylyl cyclase inhibitors before it diffused into the smooth muscle cells (Marczin et al., 1992).

In summary, in porcine tracheal smooth muscle contracted by the muscarinic receptor agonist carbachol, relaxation induced by the various NO donors may be mediated in part by cGMP and also by mechanisms independent of activation of soluble guanylyl cyclase and an increase in $[cGMP]_i$. Whereas relaxation induced by some NO donors may be associated with increases in $[cGMP]_i$, the relaxation is not completely dependent upon it. Additionally, the soluble guanylyl cyclase inhibitors methylene blue and LY83583 may attenuate relaxation induced by some NO donors independently of inhibitory effects on soluble guanylyl cyclase. Much work remains to elucidate the different mechanisms of relaxation evoked by the putative NO donors.

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