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Comparison of two soluble guanylyl cyclase inhibitors, methylene blue and ODQ, on sodium nitroprusside-induced relaxation in guinea-pig trachea

[1]Tsong-Long Hwang, [1]Chin-Chung Wu & [1,2]Che-Ming Teng

[1]Pharmacological Institute, College of Medicine, National Taiwan University, No. 1, Jen-Ai Road, Sect. 1, Taipei, Taiwan

1 To clarify further the role of cyclic GMP in mediating the relaxant response in guinea-pig trachea induced by sodium nitroprusside (SNP), the effects of soluble guanylyl cyclase inhibitors, methylene blue and 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) on SNP-induced muscle relaxation and cyclic GMP accumulation were determined.

2 SNP (0.3–100 μM) evoked a concentration-dependent relaxation of guinea-pig isolated tracheas precontracted with 0.3 μM carbachol. Preincubation of the preparations with methylene blue (10, 30 and 100 μM) resulted in a slight but concentration-dependent prevention of the relaxant response to SNP. In contrast, the relaxation to SNP was extensively prevented by 3 μM ODQ and almost abolished by 10 μM ODQ.

3 SNP (30 μM) induced a significant elevation of cyclic GMP accumulation (from 1.34 ± 0.14 to 5.39 ± 0.28 pmol mg^{-1} protein, $n=5$; $P<0.001$), which was partially attenuated by 100 μM methylene blue (3.11 ± 0.51 pmol mg^{-1} protein, $n=5$; $P<0.05$), whereas completely abolished by 10 μM ODQ (1.31 ± 0.28 pmol mg^{-1} protein, $n=5$; $P<0.001$).

4 Methylene blue, but not ODQ and N^{ω}-nitro-L-arginine methyl ester (L-NAME), caused a concentration-dependent contraction in the tracheal preparation. The tension produced by 100 μM methylene blue was $41.8 \pm 4.3\%$ (0.3 μM carbachol as 100%; $n=12$). Moreover, the non-selective muscarinic receptor antagonist atropine and the M_3-selective antagonist 4-diphenylacetoxy-N-methylpiperidine methiodine greatly inhibited the contractile effect evoked by methylene blue (100 μM).

5 In conclusion, this study provides substantial evidence that SNP-induced muscle relaxation in guinea-pig trachea is completely *via* a cyclic GMP-dependent mechanism. Furthermore, ODQ, but not methylene blue, will likely become an important tool in differentiating between cyclic GMP-dependent and -independent effects of nitric oxide.

Keywords: Sodium nitroprusside; methylene blue; ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one); guinea-pig trachea

Introduction

Soluble guanylyl cyclase (sGC) is regarded as the key enzyme in mediating tracheal relaxation induced by nitric oxide (NO) and NO-related compounds through elevating the intracellular concentration of guanosine 3′, 5′-cyclic monophosphate (cyclic GMP) (Suzuki *et al*., 1986; Watanabe *et al*., 1990; Jones *et al*., 1994; Ijima *et al*., 1995). However, not all studies support the role of sGC/cyclic GMP pathway in mediating the relaxant effect of NO-donors. Some studies have reported that there is dissociation between airway smooth muscle relaxation and cyclic GMP elevation induced by several nitrovasodilators, suggesting that the mechanism of NO-donors may involve other pathways apart from the stimulation of sGC. The controversial results of these studies may be due to different species and NO-donors used, and the lack of selective inhibitors of sGC. For example, both muscle relaxation and cyclic GMP accumulation evoked by the NO donor, sodium nitroprusside (SNP), in porcine trachea and human trachea are not inhibited by the sGC inhibitor methylene blue (Stuart-Smith *et al*., 1994; Ward *et al*., 1995). Moreover, although methylene blue inhibits SNP-induced cyclic GMP elevation, it does not attenuate SNP-induced muscle relaxation in canine trachealis (Zhou & Torphy, 1991).

In general, the sGC inhibitor most commonly used is methylene blue, but this agent has additional effects, including generation of superoxide anion (Wolin *et al*., 1990; Marczin *et al*., 1992; Kontos & Wei, 1993) and inhibition of NO synthase activity (Mayer *et al*., 1993). Furthermore, methylene blue has been reported to be rather weak in inhibition of sGC in rabbit pulmonary arterial smooth muscle cells (Marczin *et al*., 1992). Thus, these drawbacks of methylene blue may lead to misinterpretation of the role of sGC in NO-donor-mediated airway smooth muscle relaxation.

Therefore, the identification and study of cyclic GMP-dependent or -independent effects in trachea would benefit substantially from pharmacological agents that inhibit sGC selectively. Recently, a quinoxalin derivative ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one) has been described as a potent and selective inhibitor of sGC (Garthwaite *et al*., 1995; Moro *et al*., 1996). In the present study, we compared the effects of methylene blue and ODQ on cyclic GMP elevation and muscle relaxation evoked by SNP to clarify the indistinct role of sGC/cyclic GMP pathway in the NO-mediated signal transduction in guinea-pig trachea.

Methods

Mechanical responses

Male Dunkin Hartley guinea-pigs (400–500 g) were killed by a blow to the head. The trachea was excised, cleaned of adhering

[2]Author for correspondence.

fat and connective tissue, cut transversely into 4–5 rings and then opened by cutting longitudinally through the cartilage rings diametrically opposite the tracheal smooth muscle. The tracheal segment was mounted in an organ bath containing 5 ml Krebs solution of the following composition (mM): NaCl 118.2, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $CaCl_2$ 1.9 and $NaHCO_3$ 25.0. The solution was kept at 37°C and gassed with 95% O_2 plus 5% CO_2. Tracheal preparations were equilibrated for 90 min under a tension of 1 g and were washed with Krebs solution every 15 min. All experiments were carried out in the presence of indomethacin (3 μM) to prevent the formation of prostanoids and in the presence of propranolol (1 μM) to inhibit beta-adrenergic responses.

Contractions were measured isometrically with a force-displacement transducer (FT03, Grass, Quincy, MA, U.S.A.) and were recorded on a Grass Model 7DAG polygraph. After the equilibration period, the preparations were contracted with 0.3 μM carbachol for 20 min (time for tone to plateau) and then washed out for 60 min and a steady base line was restored. The contractions were induced again by 0.3 μM carbachol for 20 min and the concentration-response curves to SNP, 8-bromoguanosine 3′, 5′-cyclic monophosphate (8-Br-cyclic GMP) or forskolin were obtained. Concentration-response curves to SNP (0.3–100 μM) were obtained in the absence and in the presence of methylene blue (10, 30 or 100 μM) or ODQ (3 or 10 μM) added 40 min before the start of the curve. Concentration-response curves to 8-Br-cyclic GMP (3–300 μM) and forskolin (0.01–10 μM) were also obtained in the absence and in the presence of ODQ (10 μM) added 40 min before the start of the curve. All relaxations were expressed as per cent reversal of the carbachol-induced tension. In experiments designed to study the effect of basal production of NO on tracheal muscle contraction, tissues were precontracted with carbachol (0.3 μM) and washed out and then methylene blue (10, 30 or 100 μM), ODQ (3 or 10 μM) (for 20 min) or N^{ω}-nitro-L-arginine methyl ester (L-NAME, 300 μM; for 30 min) were added. Contractions are expressed as a percentage of the carbachol (0.3 μM)-induced contraction.

Preliminary experiments presented that methylene blue caused contraction of tracheal preparations and did not affect the maximal level of carbachol-induced tension. To examine the contractile effect of methylene blue, atropine (10 μM) and 4-diphenylacetoxy-N-methylpiperidine methiodine (4-DAMP) (10 μM) were added after a stable contraction to methylene blue (100 μM) had been obtained (about 20 min).

Cyclic GMP measurement

Tracheas prepared as above were placed in Krebs solution and continuously gassed with 95% O_2 plus 5% CO_2 at 37°C. After 5 min of incubation with SNP, the specimens were rapidly frozen in liquid nitrogen and stored at −80°C until being homogenized in 0.5 ml of 10% (w/v) trichloroacetic acid by a mechanical homogenizer. The homogenate was centrifuged at 10,000 × *g* for 5 min and the supernatant was removed and extracted with 4 × 4 volumes of water-saturated diethylether, and the cyclic GMP content was then assayed by using enzyme immunoassay kits. The pellet resuspended in 1 ml of 2 M NaOH was incubated overnight for the estimation of protein concentration by the method used by Lowry *et al.* (1951). All results are expressed as pmol cyclic GMP mg^{-1} protein.

The effects of ODQ (10 μM) and methylene blue (100 μM) on SNP-induced cyclic GMP accumulation were tested by incubating the tissues in the presence of the inhibitors for 40 min prior to the addition of SNP.

Drugs

The following drugs were used in this study: 8-Br-cyclic GMP, atropine sulphate, forskolin, indomethacin, N^{ω}-nitro-L-arginine methyl ester (L-NAME), ODQ, propranolol hydrochloride and sodium nitroprusside (Sigma Chem. Co., St. Louis, MO, U.S.A.), methylene blue (Fluka Chemie AG, Buchs, Germany), 4-DAMP (RBI, Boston, MA, U.S.A.), cyclic GMP enzyme immunoassay kit (Amersham International, Buckinghamshire, U.K.). When drugs were dissolved in dimethylsulphoxide (DMSO), the final concentration of DMSO in the bathing solution did not exceed 0.1% (v/v) and did not affect the parameters measured.

Statistical analysis

Data are presented as the means ± s.e.mean for the indicated number of separate experiments. Statistical significance was evaluated by Student's *t*-test and *P* values of less than 0.05 were considered significant.

Results

The effects of guanylyl cyclase inhibitors on SNP-induced relaxation

Carbachol produced a concentration-dependent (0.01–10 μM) contraction in guinea-pig isolated tracheas (data not shown) and the submaximal of 0.3 μM (72.4 ± 5.2%, $n = 6$) was conducted in this study. In guinea-pig isolated tracheas precontracted with carbachol (0.3 μM), the cumulative

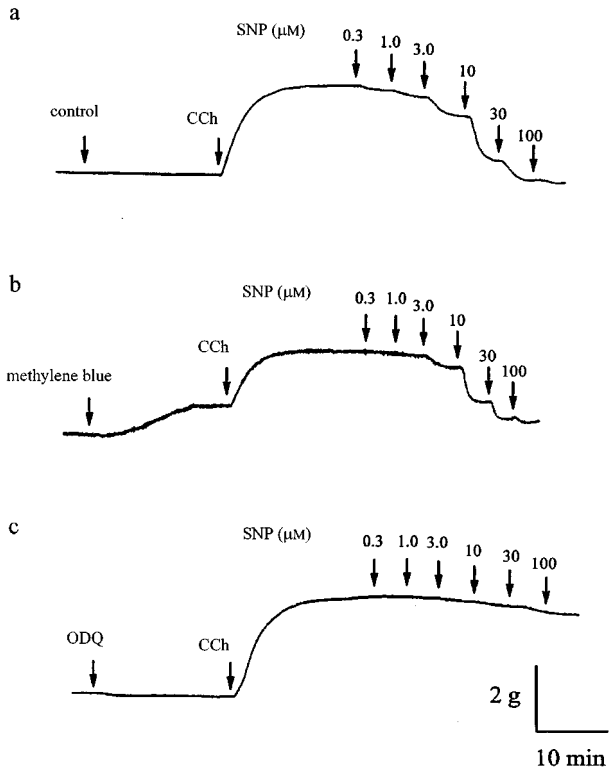

Figure 1 Concentration-response curves showing relaxations to SNP on 0.3 μM carbachol (CCh)-precontracted guinea-pig isolated tracheas and the effects of (a) control (0.1% DMSO), (b) 100 μM methylene blue or (c) 10 μM ODQ on this relaxations. Representative traces of six experiments.

applications of SNP (0.3–100 μM) caused a concentration-dependent relaxation with an EC_{50} value of 4.20 μM (Figure 1a and 2). Preincubation of the preparations with methylene blue (10, 30 and 100 μM) resulted in a slight but concentration-dependent inhibition of the relaxant response to SNP (Figure 2a); the mean inhibitory effect of methylene blue (10, 30 and 100 μM) on the relaxation caused by SNP at a maximally effective concentration (30 μM) were 7.9 ± 5.5, 9.2 ± 4.2 and $18.0 \pm 5.8\%$ ($n=6$), respectively. In contrast, 3 μM ODQ extensively prevented the relaxant effects of 30 μM SNP ($80.3 \pm 5.6\%$, $n=6$), and higher concentration of ODQ (10 μM) almost completely abolished these responses ($98.4 \pm 6.7\%$, $n=6$) (Figure 2b).

To further examine the specificity of ODQ for preventing SNP-induced relaxation, we also tested ODQ on two other known relaxant agents that signal their cellular responses independent of sGC activation. ODQ (10 μM) had no effect on relaxation of guinea-pig tracheas caused by the cyclic GMP analogue 8-Br-cyclic GMP (3–300 μM) or the adenylate cyclase activator forskolin (0.01–10 μM) (Figure 3).

The effects of methylene blue and ODQ on SNP-induced cyclic GMP accumulation

As shown in Figure 4, SNP (30 μM) caused a significant elevation of cyclic GMP accumulation as compared with the basal value (5.39 ± 0.28 pmol mg^{-1} protein and 1.34 ± 0.14 pmol mg^{-1} protein respectively, $n=5$; $P<0.001$). We chose to use 30 μM concentration of SNP in this study on the basis of relaxant experiments demonstrating that concentration to be maximally effective (Figure 2).

Methylene blue (100 μM), which alone did not affect the basal cyclic GMP level (1.25 ± 0.14 pmol mg^{-1} protein, $n=5$), partially attenuated SNP-induced cyclic GMP accumulation (3.11 ± 0.51 pmol mg^{-1} protein, $n=5$; as compared with SNP alone, $P<0.05$); on the other hand, ODQ (10 μM), which did not modify the basal cyclic GMP level (1.32 ± 0.11 pmol mg^{-1} protein, $n=5$), completely abolished cyclic GMP accumulation in response to SNP (1.31 ± 0.28 pmol mg^{-1} protein, $n=5$; as compared with SNP alone, $P<0.001$) (Figure 4).

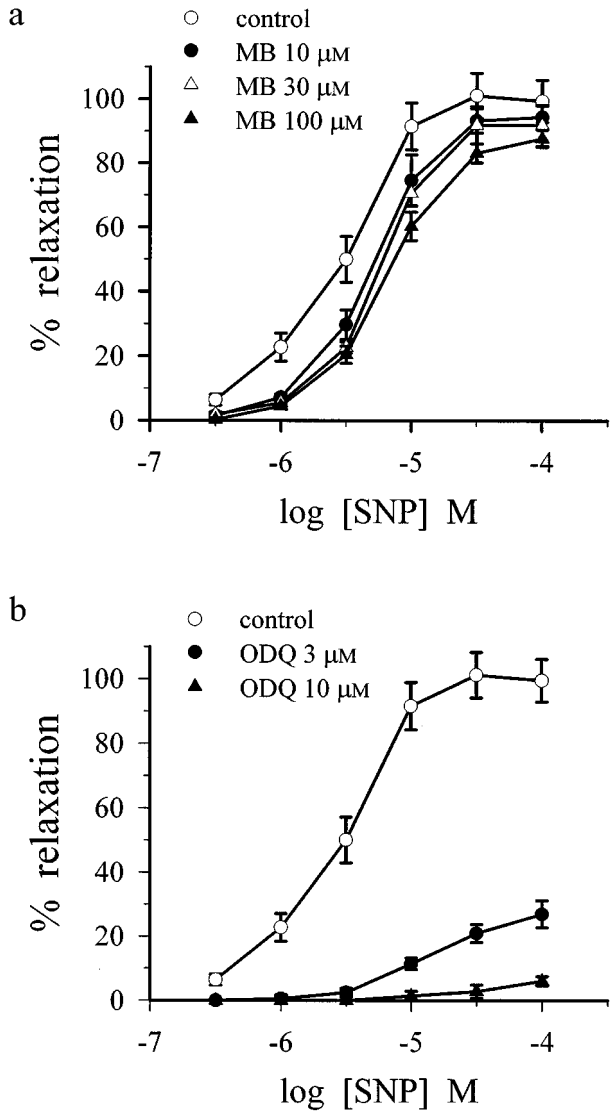

Figure 2 Concentration-response curves showing relaxations to SNP on carbachol (0.3 μM)-precontracted guinea-pig isolated tracheas and the effects of (a) methylene blue (MB) and (b) ODQ on this relaxations. Per cent relaxations are given as the means ± s.e.mean of six experiments.

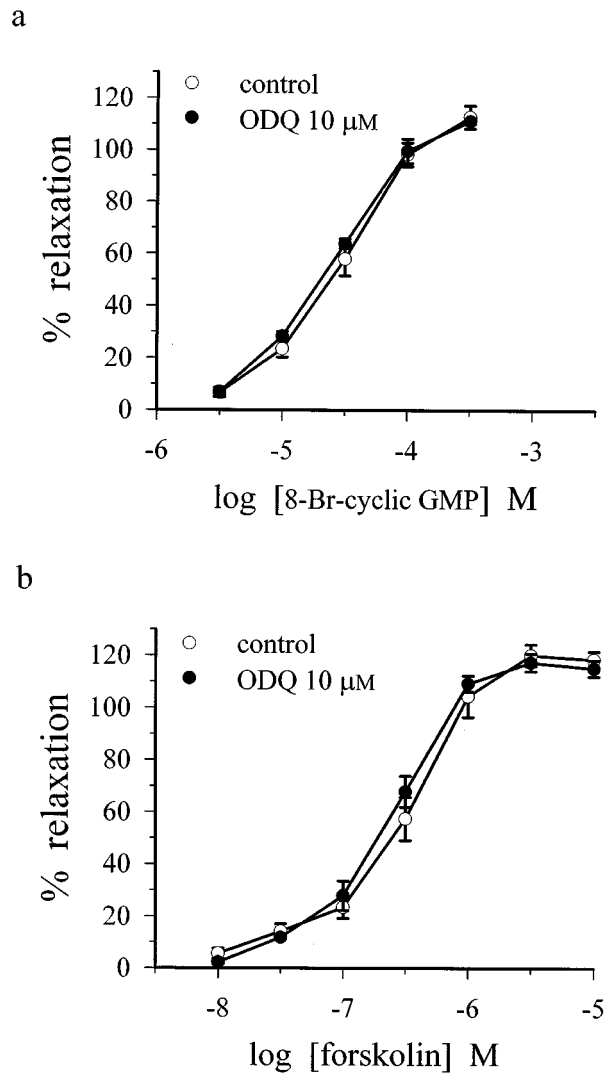

Figure 3 Concentration-response curves showing relaxations to (a) 8-Br-cyclic GMP and (b) forskolin on carbachol (0.3 μM)-precontracted guinea-pig isolated tracheas and the effects of 10 μM ODQ on this relaxations. Per cent relaxations are given as the means ± s.e.mean of six experiments.

The effects of methylene blue and ODQ on basal tension of guinea-pig trachea

Methylene blue by itself elicited a concentration-dependent contraction, whereas ODQ (10 μM) and the NO synthase inhibitor L-NAME (300 μM) did not significantly affect the basal tension in the tissue preparations (Figure 5). However, the contractile tone elicited by methylene blue (up to 100 μM) plus carbachol (0.3 μM) was not significantly different from that stimulated by carbachol alone (Figure 1). The contractions elicited by carbachol also were not altered by ODQ at a concentration up to 10 μM (Figure 1).

By using muscarinic receptor antagonists, the mechanism involved in the tracheal contraction by methylene blue was further investigated. When the non-selective muscarinic receptor antagonist atropine (10 μM) or the M_3-selective antagonist 4-DAMP (10 μM) was added to tracheas which had attained maximum contraction elicited by 100 μM methylene blue (about 20 min) significantly reversed the contraction (87.7±5.5% and 74.5±5.1% respectively, $n=6$) (Figure 6).

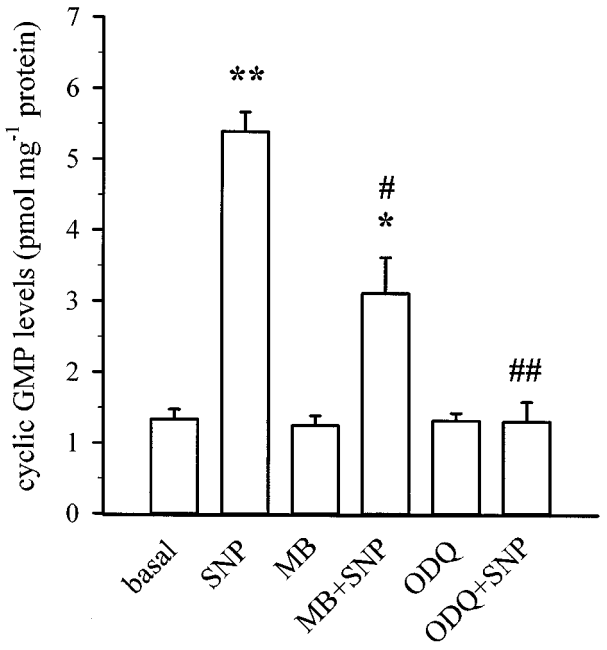

Figure 4 Cyclic GMP levels in the isolated tracheas of guinea-pig under basal condition or after treatment with SNP (30 μM), methylene blue (MB, 100 μM), methylene blue followed by SNP, ODQ (10 μM), or ODQ followed by SNP. The values are presented as the means±s.e.mean of five experiments. *$P<0.01$, **$P<0.001$ as compared with the basal value. #$P<0.05$, ##$P<0.001$ as compared with the SNP value.

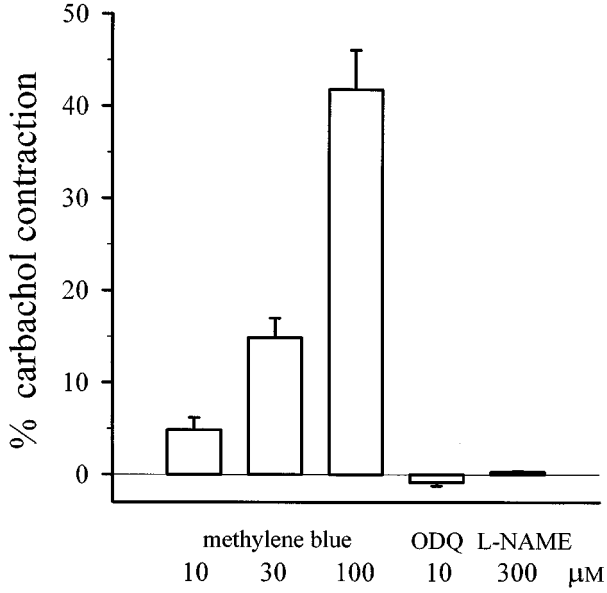

Figure 5 Effects of methylene blue (3, 10 or 100 μM, $n=12$), ODQ (10 μM, $n=6$) or L-NAME (300 μM, $n=4$) on the basal tension of isolated tracheas of guinea-pig. Tissues were precontracted with carbachol (0.3 μM) and washed out for 60 min and then test drugs were added. The values are expressed as percentages of the maximal response to carbachol (0.3 μM) and each column represents the mean±s.e.mean.

Discussion

SNP has been known for many years to evoke relaxation of tracheal smooth muscle *via* the release of NO and subsequent elevation of cyclic GMP content (Katsuki & Murad, 1977). Evidence from various studies supports the proposed role of cyclic GMP as a mediator of airway smooth muscle relaxation. For example, SNP produces a concentration-dependent relaxation of tracheal smooth muscle that is accompanied by a concentration-related increase in cyclic GMP accumulation. Moreover, the time-courses of cyclic GMP accumulation correlate closely with the relaxant action of SNP (Suzuki *et al.*, 1986; Ijima *et al.*, 1995). The principal hypothesis is that NO release from SNP occurs either spontaneously or upon photochemical reactions (Ignarro *et al.*, 1980; Arnold *et al.*, 1984). In addition, the application of 8-Br-cyclic GMP, a cell-permeant analogue of cyclic GMP, also elicits relaxation of airway smooth muscle (Suzuki *et al.*, 1986; Hamaguchi *et al.*, 1992; Sadeghi-Hashjin *et al.*, 1996a). The evidence cited above provides impressive proof of cyclic GMP as a mediator of SNP-induced relaxation in airway smooth muscle. However, there are also indications that SNP relaxes tracheal smooth

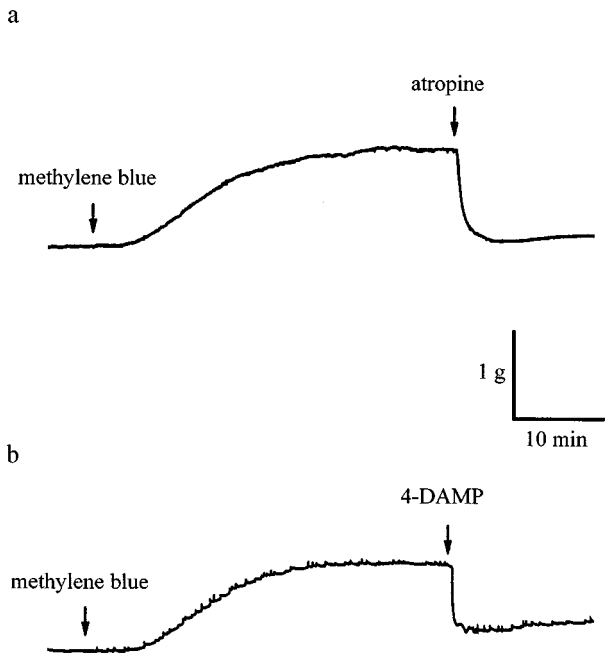

Figure 6 Inhibitory effects of (a) atropine (10 μM) or (b) 4-DAMP (10 μM) on methylene blue (100 μM)-induced contraction of guinea-pig isolated tracheas. Representative traces of six experiments.

muscle *via* a cyclic GMP-independent mechanism (Zhou & Torphy, 1991; Gaston *et al*., 1994; Stuart-Smith *et al*., 1994; Sadeghi-Hashjin *et al*., 1996b). These successive studies using methylene blue as an inhibitor of sGC to investigate the importance of cyclic GMP in SNP-induced bronchodilation led to controversial suggestions. In these studies, methylene blue has been shown either failing to modify or only partially inhibiting relaxant responses to SNP in airway smooth muscle. These results challenge the general conviction on the cyclic GMP-dependent mechanism of SNP.

Nevertheless, as discussed in the introduction, a significant limitation in all of these studies is that methylene blue is neither a powerful nor a selective inhibitor of sGC (Marczin *et al*., 1992). This implies that this compound cannot be used to distinguish between cyclic GMP-dependent and independent action of NO. In addition, such studies have often used only a particular concentration of methylene blue and have dealt, in isolation, with either cyclic GMP accumulation or muscle relaxation. Thus, the incompetence of methylene blue to prevent SNP-induced bronchodilation does not necessarily mean that sGC/cyclic GMP pathway is not involved.

Recently, the introduction of ODQ, a potent and selective inhibitor of sGC (Garthwaite *et al*., 1995), helps to identify more precisely the sGC mediated effects in various tissues (Brunner *et al*., 1996; Cellek *et al*., 1996; Moro *et al*., 1996; Ellis, 1997; Sobey & Faraci, 1997). It has been reported that ODQ neither generates superoxide anions nor inhibits NO synthase activity (Garthwaite *et al*., 1995). Furthermore, our results shown that ODQ was without effect on relaxation to the cyclic GMP analogue 8-Br-cyclic GMP or the adenylate cyclase activator forskolin in guinea-pig isolated tracheas. Taken together, these results suggest that ODQ is a potent and specific reagent for assessing the involvement of sGC in NO-mediated effects.

In the present study, we compared the effects of methylene blue and ODQ on cyclic GMP accumulation and muscle relaxation in guinea-pig trachea induced by SNP. Methylene blue up to 100 μM only shifted the concentration-relaxation curve of SNP slightly to the right. This phenomenon correlated with cyclic GMP accumulation, since methylene blue, even at the highest concentration (100 μM), only partially inhibited SNP-stimulated cyclic GMP formation. These data imply that the failure of methylene blue in preventing SNP-induced bronchodilation is not due to the sGC/cyclic GMP-independent mechanism of SNP, but the incomplete inhibition of sGC. In fact, ODQ at the concentration (10 μM) that totally inhibited SNP-stimulated cyclic GMP accumulation, also completely prevented tracheal relaxation caused by SNP. These results further support that the relaxant effect of SNP in guinea-pig trachea is mediated *via* a cyclic GMP-dependent pathway and that ODQ is a more potent sGC inhibitor than methylene blue.

Apart from the weak inhibitory effect on sGC, there is another reason to demonstrate that methylene blue is not an ideal tool in the investigation of sGC/cyclic GMP pathway in airway smooth muscle. That is, methylene blue (10 – 100 μM) by itself elicited a concentration-dependent contractile action on basal tension of guinea-pig trachea. Several lines of evidence indicated that this effect evoked by methylene blue was not under the influence of NO/cyclic GMP pathway in this study. First, although methylene blue exhibited a contractile action, this compound did not affect the basal content of cyclic GMP. Second, the more potent sGC inhibitor ODQ did not significantly alter the basal tension or cyclic GMP level. Finally, the NO synthase inhibitor L-NAME did not have any effect on basal tension. As a whole, our data also suggest that the influence of basal production of NO on muscle contraction was minor in guinea-pig trachea. The present result is consistent with that of the previous study in guinea-pig isolated, perfused tracheas (Fedan *et al*., 1995).

Recent investigations have revealed that methylene blue evokes effects other than those aforementioned, like inhibition of prostacyclin synthesis (Martin *et al*., 1989), inhibition of muscarinic receptor (Abi-Gerges *et al*., 1997; Pfaffendorf *et al*., 1997) and as a cholinesterase inhibitor. Indeed, in our preparations, all experiments were carried out in the presence of indomethacin (3 μM). Therefore, methylene blue-evoked contraction may not be mediated by the inhibition of prostanoids synthesis. In the present study, it is surprising that despite methylene blue causing a significant increase in basal tension of guinea-pig isolated tracheas, it did not affect the level of 0.3 μM carbachol-induced tension. Moreover, when tracheas were precontracted with 0.3 μM carbachol, the following addition of methylene blue (100 μM) did not further cause contraction (data not shown). These data led to speculation that methylene blue could modulate the cholinergic system in guinea-pig trachea. Interestingly, in the present investigation we showed that the increase in airway tone induced by methylene blue was inhibited by both the non-selective muscarinic antagonist atropine and the M_3-selective antagonist 4-DAMP, suggesting that the methylene blue-evoked contraction may be due to modulation of the cholinergic system in guinea-pig trachea. Such modulation may result from the augmented release of excitatory acetylcholine, the modified muscarinic receptor and/or the inhibited cholinesterase. Therefore, further studies will help to clarify how methylene blue increases basal tension in guinea-pig trachea.

In conclusion, this study provides substantial evidence that SNP-induced muscle relaxation in guinea-pig trachea is completely *via* a cyclic GMP-mediated mechanism. Methylene blue is a rather weak inhibitor of sGC and has additional effect of modulating the cholinergic system, while ODQ is a more potent and specific inhibitor of sGC than methylene blue in guinea-pig trachea. Thus, ODQ is an important tool in differentiating between cyclic GMP-dependent and -independent effects of NO.

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