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Relaxation of guinea-pig trachea by sodium nitroprusside: cyclic GMP and nitric oxide not involved

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1 Sodium nitroprusside (SNP) completely relaxed the guinea-pig isolated, perfused trachea in a concentration-dependent manner. Although SNP was less potent by about 2 orders of magnitude, its maximal effect was 25% higher compared to isoprenaline.

2 SNP (3.2 μM) increased cyclic GMP levels by 300% and relaxed guinea-pig isolated, perfused trachea by 54%. The SNP-induced relaxations of the preparations were not affected by the guanylate cyclase inhibitor, methylene blue. Moreover, zaprinast, a cyclic GMP-specific phosphodiesterase inhibitor which was supposed to enhance SNP-induced relaxations, decreased the maximal relaxation by 22% ($P<0.001$).

3 In contrast, 8Br-cyclic GMP (10 μM) increased the cyclic GMP levels by 1100% without inducing a marked relaxation.

4 SNP (10 μM) and *S*-nitroso-*N*-acetylpenicillamine (SNAP; a direct donor of nitric oxide; 10 μM), relaxed the tissues by 75% and 25%, respectively, without any nitric oxide (NO) release by SNP (<1 pmol 100 μl^{-1}), but a substantial NO release by SNAP (560 pmol 100 μl^{-1}).

5 It is concluded that the SNP-induced tracheal relaxations are probably not mediated by cyclic GMP and NO.

Keywords: Nitric oxide; nitrovasodilators; methylene blue; cyclic GMP; guanlyate cyclase; sodium nitroprusside

Introduction

The relaxant effect on airway smooth muscle of nitrovasodilators such as sodium nitroprusside (SNP) is thought to be mediated by the release of nitric oxide (NO) and elevation of guanosine 3',5'-cyclic monophosphate (cyclic GMP) (Katsuki & Murad, 1977; Murad *et al.*, 1978; Buga *et al.*, 1989; Gruetter *et al.*, 1989; Nijkamp & Folkerts, 1994). The principal activator(s) of guanylate cyclase derived from nitrovasodilators may be NO, *S*-nitrosothiol or nitrite itself (Tremblay *et al.*, 1988). These reactive intermediates are formed either spontaneously (Waldman & Murad, 1987) or through chemical reactions with cellular constituents such as sulphhydryl groups (Ignarro *et al.*, 1980). In spite of general conviction on the mechanism of action of nitrovasodilators, there is evidence in literature that mechanisms in addition to NO release may be involved in the relaxation of airways by these compounds (Stuart-Smith & Vanhoutte, 1990; Zhou & Torphy, 1991). Because of the central role of nitrovasodilators in studying NO biology, it is essential to examine the possible links and contrasts between the mechanisms of action of these chemicals and NO itself.

The aim of this study was to determine whether the proposed mechanism of action of SNP applied also to the airway smooth muscle. Concentration-response curves with isoprenaline (as a reference bronchodilator), SNP, *S*-nitroso-*N*-acetylpenicillamine (SNAP, a direct donor of NO) and 8-bromo-cyclic GMP (8Br-cyclic GMP, a cell permeable analogue of cyclic GMP) were constructed for guinea-pig isolated, perfused trachea. The effects of inhibitors of guanylate cyclase and cyclic GMP-specific phosphodiesterase on the SNP-induced relaxation were investigated. Tracheal levels of cyclic GMP were determined after adding 8Br-cyclic GMP and SNP. Moreover, tracheal relaxation by and NO generation from SNP and SNAP were compared.

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Methods

Animals

Specific pathogen-free guinea-pigs (400-500 g, male Dunkin Hartley, Harlan Olac Ltd, England) were housed under controlled conditions. Water and commercial chow were allowed *ad libitum*. The guinea-pigs were free of respiratory infections as assessed by the health monitoring quality control report by Harlan Porcellus (England), and by histological examination.

Airway responsiveness in vivo

Guinea-pigs were killed with an overdose of pentobarbitone sodium (Nembutal, 0.6 g kg^{-1} body weight, i.p.). Tracheae were dissected free of connective tissue and blood vessels, isolated, divided into two equal parts of 14 rings each and perfused in an organ bath as described before (Sadeghi-Hashjin *et al.*, 1996). Two hooks were inserted through opposite sides of the tracheal wall with the smooth muscle between them. One hook was attached to a fixed point in the organ bath; the other hook was connected to an isometric transducer (Harvard Bioscience, Kent, England). Transducers were connected to an analogue-digital convertor (Intelligent Instrumentation PCI System, Burr Brown Company, Tucson, Arizona, USA) integrating the organ baths in a semi-automatic set-up. This allowed continuous sampling, on line equilibrium detection, and real-time display of the responses on a computer screen of up to 12 baths. The tracheal tension was set at 2 g which was found to be an optimal counter weight (Nijkamp *et al.*, 1993). The inside of the trachea was perfused (2 ml min^{-1}) independently from the outside with the Krebs-bicarbonate solution by means a peristaltic pump. Krebs-bicarbonate solution was continuously gassed with 5% CO_2 in O_2 at 37°C. Every 15 min the buffer was refreshed on both sides until a stable tone was reached (usually within 75 min).

Intraluminal (IL) isoprenaline, SNP, SNAP, and 8Br-cyclic GMP concentration-response curves on isolated, perfused tracheae were constructed. In separate experiments the pre-

parations were treated with 10 μM methylene blue (IL) or zaprinast (extraluminal, EL) 25 min before application of SNP. In preliminary experiments methylene blue (as an inactivator of guanylate cyclase) and zaprinast (as a phosphodiesterase inhibitor) were found to be more effective from IL and EL sides of trachea, respectively (Sadeghi-Hashjin *et al.*, 1994). Relaxations were expressed as a percentage of the basal tension of individual preparations (approximately 2 g).

Cyclic GMP measurement

Trachea were divided into four equal segments and placed in Krebs buffer, continuously gassed with 5% CO_2 in O_2 at 37°C. The solution was refreshed three times as in the organ bath experiments. Tracheal tubes were treated with methylene blue (10 μM, 25 min), 8Br-cyclic GMP (10 μM, 15 min) or SNP (3.2 μM, 5 min; with or without methylene blue pretreatment). Thereafter, the reaction was immediately stopped with ice-cold 6% (w/v) trichloroacetic acid, the specimens were purged with liquid nitrogen and kept at −80°C. Tracheal tubes were homogenized in 6% trichloroacetic acid (100 mg ml^{-1}) by means of a mechanical homogenizator (RZR 1, Heidolph, Germany). Suspensions were centrifuged at 2000 g and 4°C for 10 min; the supernatant was preserved and the pellet was discarded. The lipid phase was extracted 4 times with 5 fold excess of water-saturated diethylether and was discarded after each time. Finally, the water phase was completely dried at 60°C under stream of nitrogen gas and the extracts were kept at −80°C until the assay was performed. For measuring cyclic GMP, the specimens were acetylated and a commercial dual-range enzymeimmunoassay (EIA) kit (RPN 226, Amersham, International plc, Buckinghamshire, UK) was used. The levels of intracellular cyclic GMP were calculated as fmol mg^{-1} wet weight of trachea.

NO measurement

NO was measured as described by Folkerts *et al.* (1995b). Krebs containing SNP or SNAP (1 pM − 100 μM) was injected into a gas stripping apparatus containing 2 ml of a 1% solution of NaI in glacial acetic acid connected to a Sievers 270B NO analyzer (Boulder, CO, U.S.A.) with a sensitivity of >1 pmol 100 μl^{-1}.

Solutions and drugs

Methylene blue was purchased from Fluka Chemie AG (Buchs, Germany). 8Br-cyclic GMP and sodium nitroprusside were bought from Sigma Chemical Co. (St Louis, U.S.A.), S-nitroso-N-acetyl-D,L-penicillamine (SNAP) from Alexis Corporation (Läufelfingen, Switzerland), and isoprenaline sulphate from OPG Groothandel B.V. (Utrecht, The Netherlands). Zaprinast (M&B 22948), a gift from Rhone-Poulenc Rorer (Essex, UK), was dissolved in ethanol as stock solution, kept at −20°C and diluted in buffer whenever needed. Krebs-bicarbonate buffer was of the following composition (mmol l^{-1}): NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 8.3.

Statistical analysis

Averaged data are presented as mean ± s.e.mean. Parameters defining the E_{max} (maximal relaxation) as well as the pD_2 value (− log of the molar concentration of a substance leading to a half E_{max}) were derived from concentration-response curves. Cyclic GMP content of the trachea was expressed as fmol mg^{-1} wet weight of the trachea. Student's unpaired *t* test was used to test the significance of the differences when two groups were to be evaluated. To examine the differences when an experiment contained more than two groups, the data were statistically evaluated with one way ANOVA, and tested group by group by Bonferroni *t* test. All *P* values <0.05 were considered to reflect a statistically significant difference.

Results

Tracheal relaxations

Isoprenaline, SNP, SNAP, and 8Br-cyclic GMP, relaxed the guinea-pig isolated, perfused trachea in a concentration-dependent manner (Figure 1). SNP ($pD_2 = 5.7 \pm 0.1$) was 80 times less potent than isoprenaline ($pD_2 = 7.6 \pm 0.2$) ($P<0.001$). However, maximal relaxation to SNP was 58% more than to 8Br-cyclicGMP ($P<0.01$) and approximately 25% more compared to isoprenaline and SNAP. The pD_2 values were not calculated for SNAP and 8Br-cyclic GMP as their curves did not reach a clear plateau at concentrations up to 100 μM.

Methylene blue did not influence the SNP-induced relaxation. In the control and treated groups, the pD_2 values were 5.65 ± 0.13 and 5.48 ± 0.24 and the maximal relaxations were $94.5 \pm 2.5\%$ and $91.8 \pm 3.2\%$, respectively (Figure 2a). Incubation with zaprinast decreased the SNP-induced maximal relaxation from $98.6 \pm 0.9\%$ to $76.5 \pm 4.7\%$ ($P<0.001$; $n=6$) without any effect on the pD_2 (Figure 2b).

Tracheal cyclic GMP levels

The basal level of cyclic GMP was 9.3 ± 2.1 fmol mg^{-1} (Figure 3a). SNP (3.2 μM) increased intracellular cyclic GMP 3 times ($P<0.05$) and relaxed the tracheae by $53.6 \pm 4.3\%$. Interestingly, 8Br-cyclic GMP (10 μM) enhanced cyclic GMP level 1100% and 200% ($P<0.001$), compared to basal and SNP-elevated cyclic GMP levels, respectively, and relaxed the tracheae only by $7.3 \pm 0.8\%$.

NO release from SNP and SNAP

SNP (10 μM) did not release NO (<1 pmol 100 μl^{-1}) and reversed the basal tension of the trachea by 77% (Figure 3b). The same concentration of SNAP released 560 pmol 100 μl^{-1}, whereas it relaxed the preparations only by 25% which was significantly less than SNP-induced relaxation ($P<0.001$; Figure 3b). At 100 μM concentrations, SNP and SNAP released 11.9 ± 11.2 and 5894 ± 125 pmol 0.1 ml^{-1} NO, respectively ($P<0.001$). However, SNP relaxed the tracheal tension by 93% and SNAP by 70% ($P<0.01$, Figure 3b).

Discussion

SNP is known as a potent vasodilator that causes smooth muscle relaxation by releasing NO which activates the cyto-

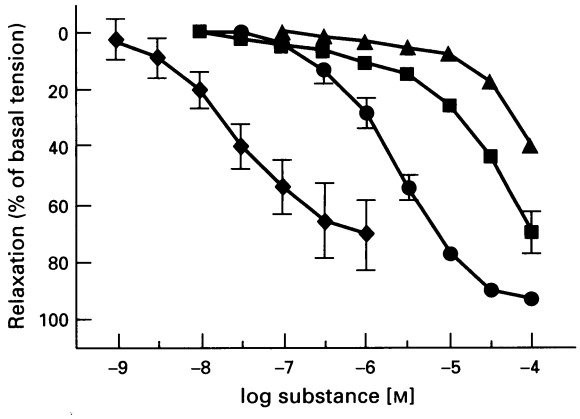

Figure 1 Intraluminal (IL) isoprenaline (◆), SNP (●), SNAP (■), and 8Br-cyclic GMP (▲) concentration-response curves of the guinea-pig isolated, perfused trachea. The responses are expressed as percentage relaxation of the basal tension (approximately 2 g). Isoprenaline was the most potent substance and SNP caused the highest maximal relaxation at the concentration applied. $n=4-6$.

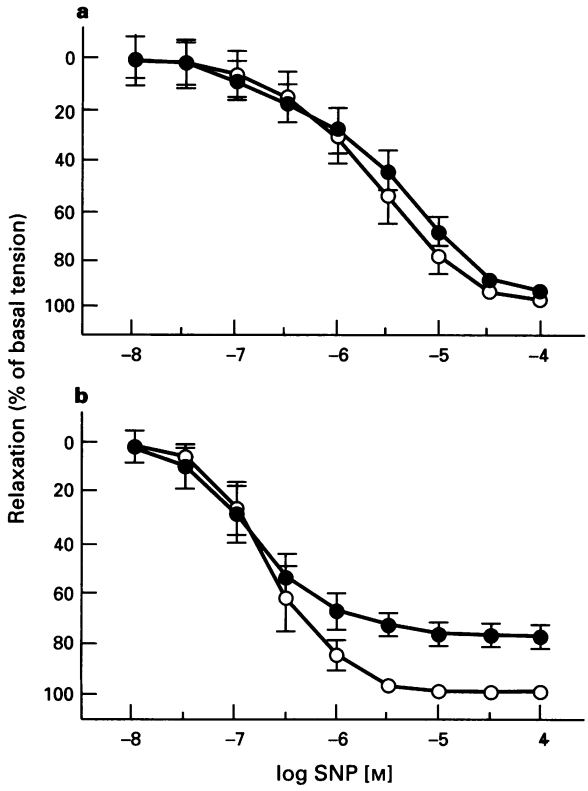

Figure 2 Effects of methylene blue (10^{-5} M, a) and zaprinast (10^{-5} M, b) on sodium nitroprusside (SNP) concentration-response curves of the guinea-pig isolated, perfused trachea. The responses are expressed as percentage relaxation of the basal tension (approximately 2 g). Methylene blue and zaprinast were added to half of the baths 25 min before the concentration-response determination. Responses of the control (○) and treated tracheae (●); $n=6$.

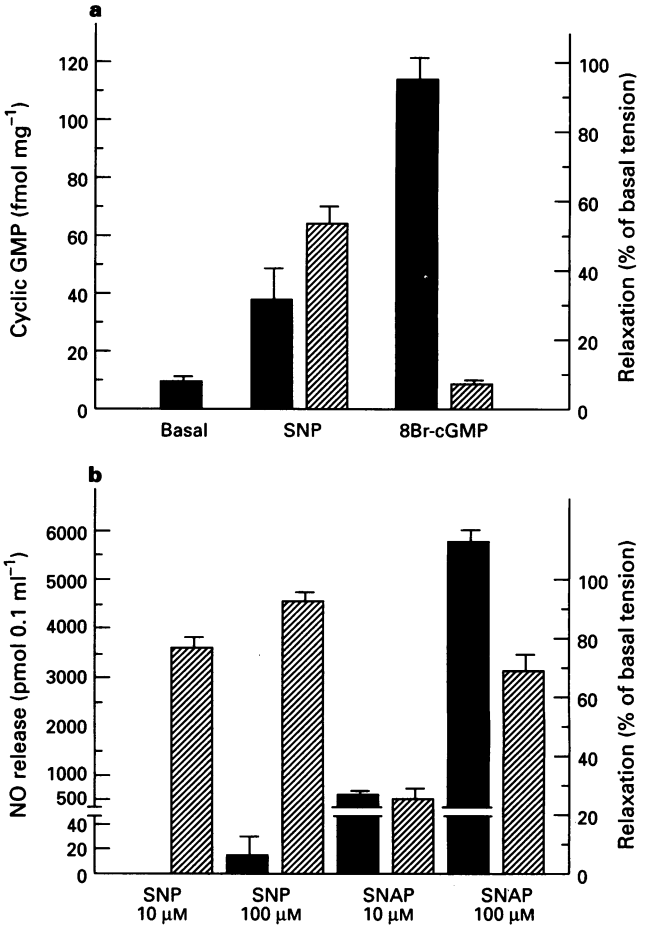

Figure 3 (a) Cyclic GMP levels (solid columns) and relaxations (hatched columns) after exposure of guinea-pig trachea to SNP (3.2 μM) and 8Br-cyclic GMP (10 μM). There was apparently no correlation between cyclic GMP elevation and relaxation induced by these substances. $n=3-6$. (b) The amount of nitric oxide released from SNP and SNAP (solid columns) and tracheal relaxation by these nitrovasodilators (hatched columns). The relaxation induced by SNP was more than by SNAP ($P<0.01$), whereas SNP released no detectable or very small amount of NO. $n=4-6$.

solic isozyme of guanylate cyclase (Ignarro & Kadowitz, 1985). The leading theory is that NO release from SNP occurs either spontaneously or upon photochemical reactions (Ignarro *et al.*, 1980; Arnold *et al.*, 1984). However, there are also indications that SNP relaxes smooth muscle via a cyclic GMP-independent mechanism (Diamond, 1993; Gaston *et al.*, 1994). It was shown that SNP increased intracellular calcium concentration in a murine macrophage cell line (Kong *et al.*, 1994). This opposes the current opinion that guanylate cyclase activators decrease $[Ca^{2+}]_i$ via activation of protein kinase G and Ca^{2+}-ATPase (Diamond, 1993).

The ability of methylene blue to inactivate guanylate cyclase has long been appreciated (Martin *et al.*, 1985). Interestingly, in the present study, methylene blue did not influence the tracheal responsiveness to SNP at all. Furthermore, in a concentration that clearly relaxed the trachea, SNP failed to release NO and it had a significantly higher relaxant potency compared with SNAP which released 500 times more NO. Nano- and micromolar concentrations of NO are sufficient to modulate airway smooth muscle responses (Buga *et al.*, 1989; Folkerts *et al.*, 1995a). Therefore, the detection range of our NO-analyzer was sufficient for the purpose of this study.

The role of cyclic GMP as a mediator of relaxation was examined in this study by applying 8Br-cyclic GMP. This analogue has been shown to diffuse into the cells and to reverse spontaneous tone of guinea-pig trachea (Suzuki *et al.*, 1986). In our study, the analogue induced a concentration-dependent relaxation of guinea-pig trachea and increased cyclic GMP level. However, an increased cyclic GMP level of more than 10 fold was accompanied by only a small relaxation. SNP-induced relaxation was significantly more than with 8Br-cyclic GMP with a smaller increase of cyclic GMP level. A selective inhibitor of cyclic GMP phosphodiesterase, zaprinast, relaxes bovine tracheal smooth muscle (Shahid *et al.*, 1991), potentiates sodium nitroprusside-induced relaxation and causes cyclic GMP accumulation in canine trachea (Zhou & Torphy, 1991; Torphy *et al.*, 1991). In the present study, the SNP-induced relaxation of guinea-pig trachea was decreased after treatment with zaprinast. Whatever mechanism was involved behind this effect of zaprinast, the experiment suggested that SNP may relax the airways of guinea-pigs via an alternative pathway than guanylate cyclase system.

A previous study has suggested that SNP released NO only in the presence of vascular tissue (Bates *et al.*, 1991). However, in our study the presence of tissue did not increase NO liberation from SNP (data not shown). The species and organ differences, i.e. guinea-pig trachea vs. rabbit aorta, may explain this contradiction. Furthermore, the amount of NO, released from 1 mM SNP in the presence of vascular tissue, was only about 9.5 pmol ml^{-1} s^{-1} (Bates *et al.*, 1991). As shown in our study, a NO level as high as 560 pmol 0.1 ml^{-1} (released from 10 μM SNAP) was required to relax the trachea by 25%.

Zhang *et al.* (1993) showed that relaxation of guinea-pig trachea by SNP was decreased by methylene blue and potentiated by zaprinast. Some differences in the experimental protocols could be responsible for these different observations. Zhang *et al.* (1993), for instance, used tracheal rings whereas in

the present study intact tubes were used. Fedan *et al.* (1995) observed a significant inhibition by methylene blue of the guinea-pig pre-contracted, perfused trachea and expressed the results as percentage of methacholine response. It is likely that, after incubation with methylene blue, the contractile response to methacholine also changes and complicates the relaxation process as well. In agreement with our findings, Stuart-Smith and co-workers (1994) showed that the relaxation to NO but not to SNP of porcine airway muscle was inhibited by methylene blue. Tissues pretreated with methylene blue that failed to relax to NO were, however, relaxed by SNP demonstrating that SNP relaxes airways by a mechanism other than or in addition to the release of NO. However, in contrast to our experiments, in none of the above mentioned reports were the functional studies accompanied by investigation of NO release from SNP or cyclic GMP elevation by this agent. Zhou & Torphy (1991) combined functional and biochemical studies and reported that methylene blue inhibited SNP-induced cyclic GMP accumulation in canine trachea but, paradoxically, potentiated its relaxation. The debate on the mechanism of action of SNP is not limited to airway smooth muscle. For example, elevation of cyclic GMP levels in rat vas deferens induced by SNP is not always associated with relaxation (Diamond & Janis, 1978). Even in the vasculature, there appears to be a slight dissociation between relaxation and cyclic GMP elevation by some nitrovasodilators (Axelsson *et al.*, 1982; Diamond & Chu, 1983). Thus, in spite of therapeutic application for over 6 decades, the role of cyclic GMP in the actions of SNP is not resolved.

In conclusion, the SNP-induced guinea-pig tracheal relaxation is not mediated by cyclic GMP and NO. SNP is usually used in the (bio)medical field as an approved NO donor and activator of guanylate cyclase. This general belief has to be explored more precisely to prevent misinterpretations due to false assumptions.

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