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YC-1 potentiates nitric oxide-induced relaxation in guinea-pig trachea

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1 The effects of YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole) on tension, levels of cyclic GMP and cyclic AMP were investigated in guinea-pig trachea. We especially studied the combined effect of YC-1 with exogenous or endogenous nitric oxide on these parameters.

2 YC-1 at the concentration 3 or 10 μM, which caused only minor effect by itself, elicited concentration-dependent potentiation of sodium nitroprusside (SNP)-induced tracheal relaxation. This relaxation of YC-1 with SNP was reversed by ODQ.

3 Relaxant responses to electric field stimulation (EFS) in the presence of indomethacin, atropine, guanethidine, α-chymotrypsin and histamine were also markedly increased by YC-1 (10 μM). In the presence of L-NAME or ODQ, the relaxant effects to EFS were attenuated and the following addition of YC-1 did not further enhance relaxation.

4 YC-1 (10 μM) or SNP (0.3 μM) alone did not induce significant elevation of cyclic GMP levels in the presence of IBMX, whereas simultaneous application of both compounds markedly elevated the cyclic GMP accumulation. In contrast, the cyclic AMP levels were not altered even at the combination of YC-1 and SNP. Additionally, YC-1 also affected cyclic GMP metabolism, since it inhibited the activity of phosphodiesterase type V in human platelets.

5 YC-1 (30 μM) did not scavenge superoxide anion and had no effect on the removal of superoxide anion by superoxide dismutase in a xanthine/xanthine oxidase system.

6 In conclusion, these results indicate that although YC-1 elicits negligible relaxation of guinea-pig trachea by itself, it can potentiate the relaxant responses of exogenous or endogenous NO. This synergistic response of YC-1 is *via* the elevation of cyclic GMP contents.

Keywords: cyclic GMP; guinea-pig trachea; nitric oxide; ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one); YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole); phosphodiesterase

Abbreviations: cyclic AMP, adenosine 3′,5′-cyclic monophosphate; cyclic GMP, guanosine 3′,5′-cyclic monophosphate; DMSO, dimethylsulphoxide; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole)

Introduction

A growing comprehension of the involvement of nitric oxide (NO) in numerous bioregulatory pathways has not only expanded new therapeutic avenues for NO-related compounds but also led to an increased use of such compounds in pharmacological studies. Soluble guanylyl cyclase (sGC), a haeme-containing enzyme, plays a pivotal role in the transduction of inter- and intra-cellular signals conveyed by the signal molecule NO. By the formation of guanosine 3′, 5′-cyclic monophosphate (cyclic GMP), this enzyme mediates NO-mediated actions such as vascular smooth muscle relaxation, inhibition of platelet aggregation and synaptic transmission (Moncada & Higgs, 1995). Besides, NO has also been demonstrated to be the primary relaxant transmitter in human airways (Belvisi *et al*., 1992). sGC is regarded as the key enzyme in mediating tracheal relaxation induced by NO and NO-related compounds through elevating the intracellular concentration of cyclic GMP (Suzuki *et al*., 1986; Watanabe *et al*., 1990; Jones *et al*., 1994; Ijima *et al*., 1995). However, there are also indications that NO-donors relax tracheal smooth muscle *via* a cyclic GMP-independent mechanism (Zhou & Torphy, 1991; Gaston *et al*., 1994; Stuart-Smith *et al*., 1994; Sadeghi-Hashjin *et al*., 1996a). Until recently, we (Hwang *et al*., 1998) and others (Ellis, 1997) have shown that the relaxant effect of NO was abolished by ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one), a selective inhibitor of sGC (Garthwaite *et al*., 1995; Moro *et al*., 1996), suggesting that the effect was mediated exclusively by activation of sGC and accumulation of intracellular cyclic GMP.

YC-1, a chemically synthetic benzylindazol derivative (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole), has been demonstrated as an inhibitor of platelet aggregation in our laboratory (Ko *et al*., 1994; Wu *et al*., 1995). This substance also attenuated platelet-rich thrombosis *in vivo* (Teng *et al*., 1997). These effects were caused by elevation of cyclic GMP levels *via* a NO-independently activation of sGC. In addition, it has been reported that YC-1 stimulates sGC directly and increases the responsiveness of the enzyme towards NO and CO (Friebe *et al*., 1996). Furthermore, the synergistic responses to YC-1 with NO-donors in cultured vascular smooth muscle cells and isolated aortic rings also have been demonstrated (Mülsch *et al*., 1997). However, the activity of YC-1 in tracheal relaxation has not been characterized. In this study, we investigate the effects of YC-1 on tension, levels of cyclic GMP and cyclic AMP in guinea-pig trachea, as well as the combined effect of YC-1 with exogenously added or endogenously released NO on these parameters. Our data suggested that YC-1 could increase the relaxant effect of NO

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via the synergistic elevation of cyclic GMP levels in guinea-pig trachea.

Methods

Mechanical responses

Male Dunkin Hartley guinea-pig (400–500 g) was killed by a blow to the head. The trachea was excised, cleaned of adhering 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 segments were 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. Contraction was measured isometrically with a force-displacement transducer (FT03, Grass, Quincy, MA, U.S.A.) and recorded on a Grass Model 7DAG polygraph. The preparation was allowed to equilibrate for 90 min under a tension of 1 g and washed with Krebs solution every 15 min.

Tissues were pretreated with indomethacin (3 μM) and propranolol (1 μM) to prevent the formation of prostanoids and to inhibit beta-adrenergic responses, respectively. Then 0.3 μM carbachol was administered and once a stable contraction was obtained, the concentration-response curves to YC-1 and SNP were presented. In order to assess the combined effect of YC-1 and SNP, YC-1 (3 or 10 μM) was added 15 min before the start of the concentration-response curves to SNP. Next, to examine whether the potentiating effects of YC-1 are affected by zaprinast, a selective inhibitor of phosphodiesterase type V, we investigated the action of YC-1 on SNP-induced relaxation in the presence of zaprinast (30 μM). All relaxations were expressed as per cent reversal of the carbachol-induced tension.

Both NO and VIP have been shown to be inhibitory non-adrenergic non-cholinergic (iNANC) transmitters in guinea-pig trachea (Ellis & Farmer, 1989; Tucker *et al*., 1990). The NO component is inhibited by NO synthase inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME), whereas the VIP component is inhibited by the peptidase α-chymotrypsin (Ellis & Farmer, 1989). In a separate series of experiments, to study the effect of YC-1 on the nitrergic relaxation, transmural electrical stimulation (50 V, 0.5 ms) was performed *via* two platinum plate electrodes by a Grass S88 stimulator. In all experiments, indomethacin (3 μM), atropine (3 μM) and guanethidine (3 μM) were present continuously in the bathing solution to prevent the formation of prostanoids and to block cholinergic and adrenergic responses, respectively. The effects of YC-1 (10 μM) on electrically induced-iNANC neurotrans-

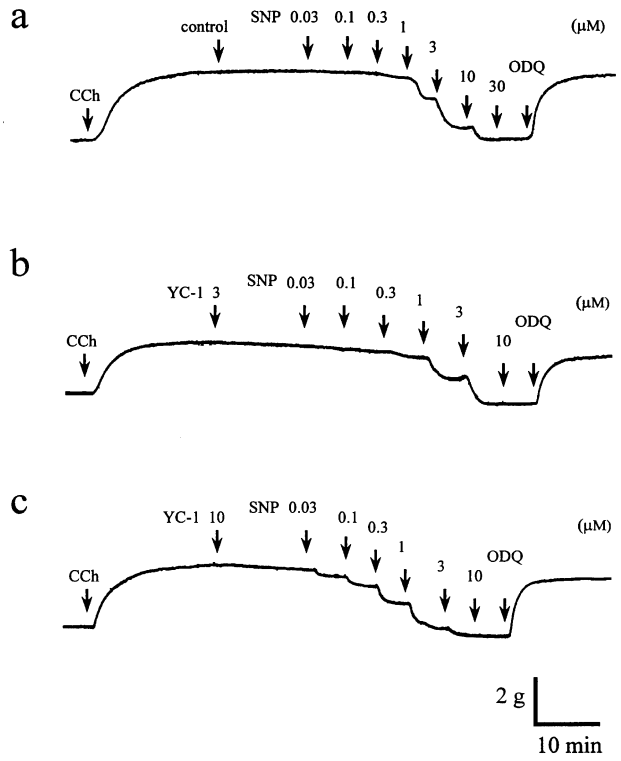

Figure 1 Concentration-dependent relaxation to SNP in guinea-pig isolated trachea and the effect of (a) control (0.1% DMSO), (b) 3 μM YC-1 or (c) 10 μMYC-1 on this relaxation. Tissues were treated with indomethacin (3 μM) and propranolol (1 μM) and contracted with 0.3 μM carbachol (CCh). ODQ (10 μM) was added to tissues following the establishments of a stable maximal response to SNP. Representative traces of 5–6 experiments.

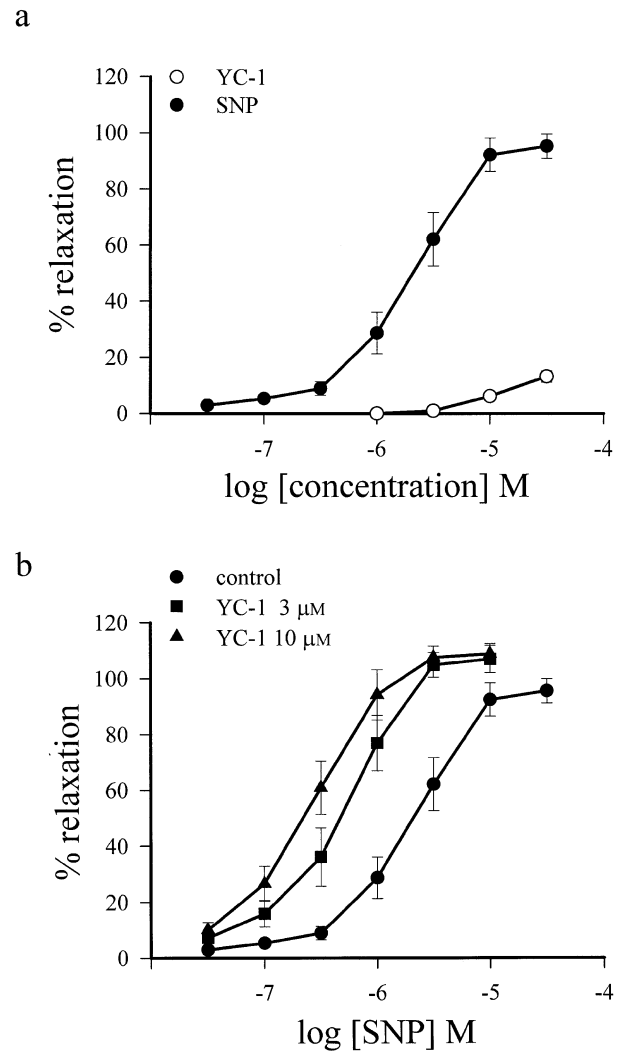

Figure 2 Potential relaxation of YC-1 and SNP on 0.3 μM carbachol-precontracted guinea-pig isolated trachea. Tissues were treated with indomethacin (3 μM) and propranolol (1 μM). (a) Concentration-response curves to YC-1 or SNP. (b) Concentration-response curves to SNP in the absence (control; 0.1% DMSO) or presence of YC-1 (3 μM or 10 μM). Per cent relaxation is given as the mean ± s.e.mean of 5–6 experiments.

mitters were studied as follows. After the equilibration period, α-chymotrypsin (2 U ml^{-1}) was added to inhibit the VIP component of the iNANC response. Tissues were then contracted with histamine (10 μM) and the effect of YC-1 on frequency-dependent relaxation (1–16 Hz) was obtained in the absence or presence of L-NAME (300 μM) or ODQ (10 μM). To examine the specific effect of YC-1 on NO-induced relaxation, tissues were treated initially with L-NAME (300 μM) instead of α-chymotrypsin to inhibit the NO component of the iNANC response. Then frequency-dependent responses (2–32 Hz) were performed in the absence or presence of YC-1 (10 μM). The iNANC responses were completely blocked by tetrodoxin (3 μM) confirming that they were due to nerve stimulation. All relaxations were expressed as per cent reversal of the histamine-induced tension.

Cyclic nucleotides measurement

Trachea prepared as above was placed in Krebs solution and continuously gassed with 95% O_2 plus 5% CO_2 at 37°C. After 30 min of incubation with phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 300 μM), SNP (0.3 μM; for 5 min), YC-1 (10 μM; for 20 min) or both was added. In some experiments, ODQ (10 μM) was also presented in the incubation buffer. The specimens were then rapidly frozen in liquid nitrogen and stored at −80°C until being homogenized in 0.5 ml of 10% (w v^{-1}) 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 and cyclic AMP content were 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). Cyclic GMP and cyclic AMP values are presented as pmol mg^{-1} protein.

Phosphodiesterase assay

The method of Hidaka & Asano (1976) was used. Washed human platelets prepared as previously described (Huang *et al.*, 1991) were resuspended in 50 mM Tris-HCl (pH 7.5,

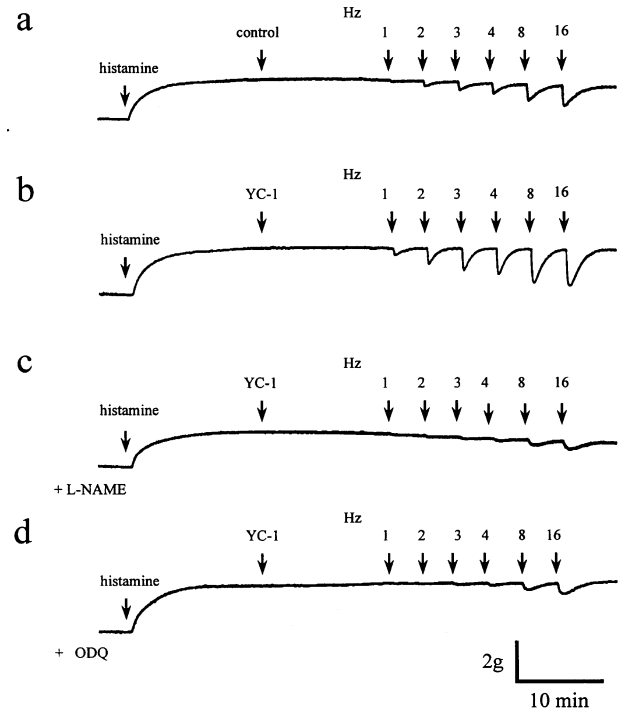

Figure 3 Effect of YC-1 on the relaxation elicited by field stimulation (1–16 Hz, 50 V, 0.5 ms for 10 s) in guinea-pig isolated trachea. Tissues were treated with indomethacin (3 μM), atropine (3 μM), guanethidine (3 μM) and α-chymotrypsin (2 U ml^{-1}) and contracted with histamine (10 μM). Original tracings showing the relaxation (a) in the absence (control; 0.1% DMSO) or (b) presence of YC-1 (10 μM). In some, experiments were performed in the preincubation with (c) L-NAME (300 μM) or (d) ODQ (10 μM). Representative traces of six experiments.

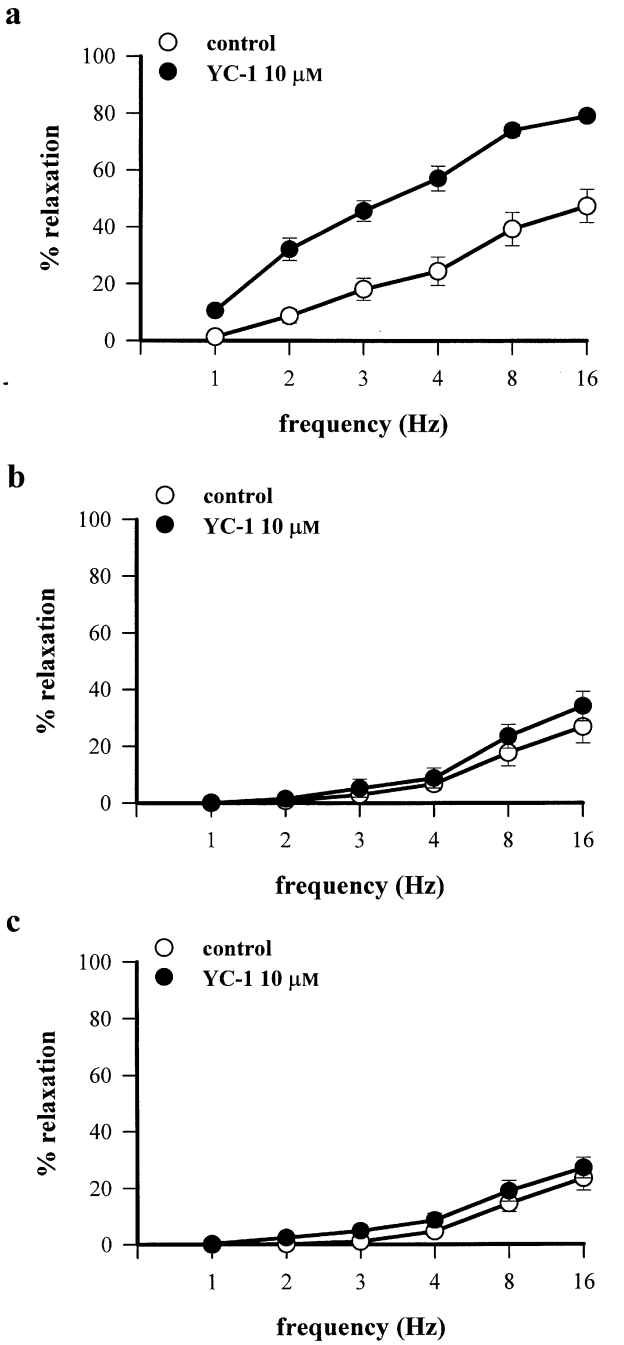

Figure 4 Potential effect of YC-1 on the relaxation elicited by field stimulation (1–16 Hz, 50 V, 0.5 ms for 10 s) in guinea-pig isolated trachea. Tissues were treated with indomethacin (3 μM), atropine (3 μM), guanethidine (3 μM) and α-chymotrypsin (2 U ml^{-1}) and contracted with histamine (10 μM). Experiments were performed in the preincubation with (a) solvent (b) ODQ (10 μM) or (c) L-NAME (300 μM). Per cent relaxation is given as the mean ± s.e.mean of six experiments.

containing 5 mM $MgCl_2$). Platelets were then disrupted by sonication, and soluble phosphodiesterase preparation was obtained by ultracentrifugation $105{,}000 \times g$ for 60 min (4°C).

The enzyme (11.7 mg ml^{-1}; 10 μl) was incubated with Tris-HCl (80 μl) and 10 μM cyclic GMP substrate (final concentration 1 μM containing 0.1 μCi [^{3}H]-cyclic GMP) was added. After 20 min at 30°C, the samples were heated to 100°C for 2 min before cooling. *Ophiophagus hannah* snake venom (10 mg ml^{-1}; 10 μl) was then added and incubated at 30°C for 10 min to convert the 5′-GMP to the uncharged nucleosides, guanosine. An ion-exchange resin (200 μl) was added to bind all unconverted cyclic GMP. After centrifuging, the supernatant was removed for determination in a liquid scintillation counter.

Superoxide anion radical (O_2^-) scavenging activity

Since O_2^- may inactivate NO/sGC-induced tracheal relaxation (Sadeghi-Hashjin *et al*., 1996a), the method of SOD-inhibitable cytochrome *c* reduction was utilized to investigate the effect of YC-1 on the O_2^- generation. The O_2^- scavenging activity of YC-1 was determined by monitoring their competition with cytochrome *c* for O_2^- generated by xanthine/xanthine oxidase system (Fridovich, 1970). YC-1 (30 μM) was incubated in KH_2PO_4/K_2HPO_4 buffer (50 mM; pH 7.4) containing cytochrome *c* (0.5 mg ml^{-1}), K_2H_2-EDTA (0.3 mM), xanthine (100 μM) and 0.02 U ml^{-1} xanthine oxidase. Reduction of cytochrome *c* was measured spectrophotometrically at 550 nm at 25°C. The first-minute rate of O_2^--induced cytochrome *c* reduction in the presence of solvent of YC-1 (0.1% DMSO) was taken as 100%. Superoxide dismutase (SOD; 20 U ml^{-1}) was used as a positive control.

Drugs

YC-1 was chemically synthesized as described previously (Yoshina & Kuo, 1978) and was dissolved in dimethylsulphoxide (DMSO) for a stock solution. α-Chymotrypsin, atropine sulphate, carbachol, cyclic GMP, forskolin, guanethidine, histamine dihydrochloride, IBMX, indomethacin, L-NAME, ODQ, propranolol hydrochloride, sodium nitroprusside, xanthine oxidase and zaprinast were purchased from Sigma (St. Louis, MO, U.S.A.), cyclic AMP and cyclic GMP enzyme immunoassay kit and [^{3}H]-cyclic GMP was from Amersham (Buckinghamshire, U.K). When drugs were dissolved in DMSO, the final concentration of DMSO in the bathing solution did not exceed 0.4% (v v^{-1}) and did not affect the parameters measured.

Statistical analysis

Data are presented as the mean ± s.e.mean for the indicated number of separate experiments. Statistical analysis was performed by ANOVA followed by the Dunnett's test and *P* values of less than 0.05 were considered significant.

Results

The effect of YC-1 on NO-induced tracheal relaxation

SNP (0.03 – 30 μM) elicited concentration-dependent relaxation of tracheal segments prestimulated with carbachol (0.3 μM), which was almost completely reversed by 10 μM ODQ (Figures 1a and 2). In contrast, YC-1 caused only slight tracheal relaxation at concentrations up to 30 μM (Figure 2a). To study the possible synergistic effects of YC-1 and NO-donor at the tracheal relaxation, the effect of YC-1-pretreatment on SNP-induced relaxation was investigated.

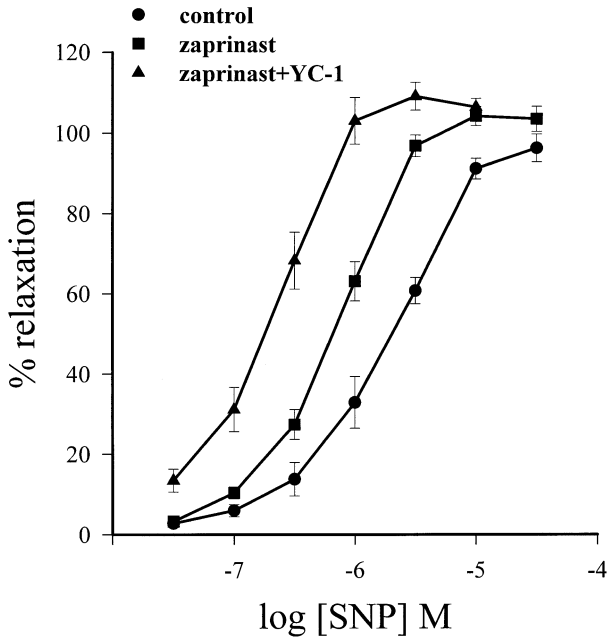

Figure 5 Effects of zaprinast (30 μM) on relaxation induced by cumulative concentrations of SNP in the absence or presence of YC-1 (10 μM) in guinea-pig isolated trachea. Tissues were treated with indomethacin (3 μM) and propranolol (1 μM) and contracted with 0.3 μM carbachol (CCh). Per cent relaxation is given as the mean ± s.e.mean of six experiments.

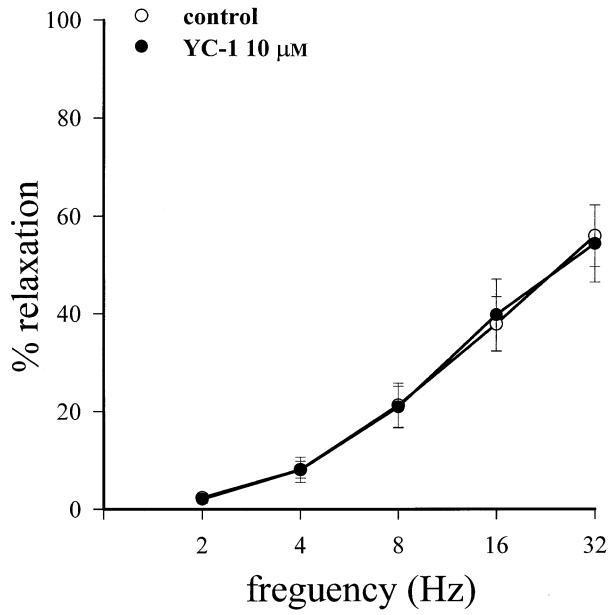

Figure 6 Effect of YC-1 on the relaxation elicited by field stimulation (2 – 32 Hz, 50 V, 0.5 ms for 10 s) in guinea-pig isolated trachea. Tissues were treated with indomethacin (3 μM), atropine (3 μM), guanethidine (3 μM) and L-NAME (300 μM) and contracted with histamine (10 μM). Experiments were performed in the absence (control; 0.1% DMSO) or presence of YC-1 (10 μM). Per cent relaxation is given as the mean ± s.e.mean of six experiments.

YC-1, at the concentration 3 or 10 μM, which caused only minor effect, elicited concentration-dependent potentiation of SNP-induced relaxation (Figures 1 and 2). These potentiating effects of YC-1 were approximately 5 and 10 fold, respectively. The half-maximal relaxation (EC_{50}) to SNP was 2.60 ± 0.87 μM ($n=6$) in the absence of YC-1 and 0.49 ± 0.13 μM ($n=5$; $P<0.05$) or 0.25 ± 0.06 μM ($n=6$; $P<0.05$) in the presence of YC-1 3 or 10 μM, respectively. ODQ (10 μM) also reversed the relaxant effects of YC-1 together with SNP (Figure 1b,c).

To assess the effect of YC-1 on the nitrergic relaxation, the effect of YC-1 on the response to field stimulation was investigated. In histamine (10 μM)-precontracted trachea, electrical field stimulation (1–16 Hz) in the presence of atropine, guanethidine, indomethacin (30 μM) and α-chymotrypsin (2 U ml^{-1}) evoked rapid, short-lasting relaxation, which was significantly attenuated by the preincubation of L-NAME (300 μM) or ODQ (10 μM) (Figures 3 and 4). YC-1 (10 μM) caused a leftward-shift in the frequency-dependent relaxation; however, when trachea was preincubated with L-NAME (300 μM) or ODQ (10 μM), the following addition of YC-1 did not further enhance the relaxation (Figures 3 and 4).

To examine whether the potentiating effects of YC-1 are affected by zaprinast, we investigated the action of YC-1 on SNP-induced relaxation in the presence of zaprinast. Preincubation with zaprinast (30 μM) caused a leftward-shift in the SNP-induced relaxation (Figure 5). The EC_{50} values to SNP in the absence and presence of zaprinast were 2.25 ± 0.11 μM ($n=6$) and 0.68 ± 0.07 μM ($n=12$; $P<0.05$), respectively. Moreover, the addition of YC-1 further enhanced the SNP-induced relaxation in the presence of zaprinast with an EC_{50} value of 0.18 ± 0.05 ($n=6$; $P<0.05$). These effects of zaprinast in the absence and presence of YC-1 were approximately 3 and 13 fold, respectively.

To further examine the specificity of YC-1 for increasing NO-induced relaxation, we also examined the effect of YC-1 on tissues that were treated with L-NAME (300 μM) instead of α-chymotrypsin. The preparations responded to field stimulation (2–32 Hz) with relaxation, although the duration was longer than that observed in the presence of α-chymotrypsin. The relaxant responses to stimulation after incubation of tracheal strips with L-NAME were not affected by 10 μM YC-1 (Figure 6). YC-1 (10 μM) had no effect on relaxation caused by the adenylate cyclase activator forskolin (0.01–1 μM) in guinea-pig trachea precontracted with 0.3 μM carbachol (Figure 7). Furthermore, we examined the effects of YC-1 on atrial natriuretic peptide (ANP)-induced relaxation. In guinea-pig isolated trachea precontracted with histamine (10 μM), the cumulative applications of ANP (0.003–1 μM) caused a concentration-dependent relaxation. Preincubation of the preparations with YC-1 (10 μM) resulted in a slight potentiation of the relaxant response to ANP (Figure 8). This potentiating effect of YC-1 was only 2 fold. The EC_{50} value to ANP was 0.39 ± 0.16 μM ($n=6$) in the absence of YC-1 and 0.19 ± 0.05 μM ($n=6$) in the presence of YC-1, respectively. This result suggests that YC-1 is more specific for SNP-induced relaxation; whereas, the effect of YC-1 led to 10 fold increase.

The effects of YC-1 on cyclic GMP and cyclic AMP accumulation

In the presence of nonselective PDE inhibitor IBMX (300 μM), SNP (0.3 μM) or YC-1 (10 μM) applied alone did not elicit significant elevation of cyclic GMP accumulation as compared with the basal value (5.81 ± 0.67, 7.02 ± 1.15 and 4.62 ± 0.63 pmol mg^{-1} protein, respectively; $n=4$); however, YC-1 together with SNP synergistically elevated the cyclic GMP levels (11.33 ± 0.89 pmol mg^{-1} protein; $n=4$; $P<0.001$) (Figure 9). The increase of cyclic GMP levels caused by SNP and YC-1 was abolished by the preincubation with 10 μM ODQ (4.00 ± 0.47 pmol mg^{-1} protein; $n=4$) (Figure 9). We chose to use 0.3 μM SNP and 10 μM YC-1 in this study on the basis of relaxant experiments, demonstrating that these concentrations have a minor effect (Figure 2a). Additionally, the cyclic AMP levels were not altered even at the combination of these two compounds (Figure 9).

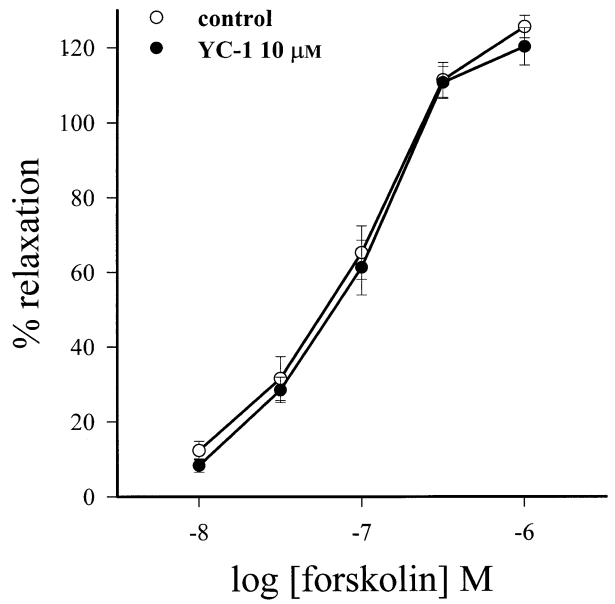

Figure 7 Concentration-response curves showing relaxation to forskolin in guinea-pig isolated trachea and the effects of YC-1 (10 μM) on this relaxation. Tissues were treated with indomethacin (3 μM) and propranolol (1 μM) and contracted with 0.3 μM carbachol (CCh). Per cent relaxation is given as the mean ± s.e.mean of six experiments.

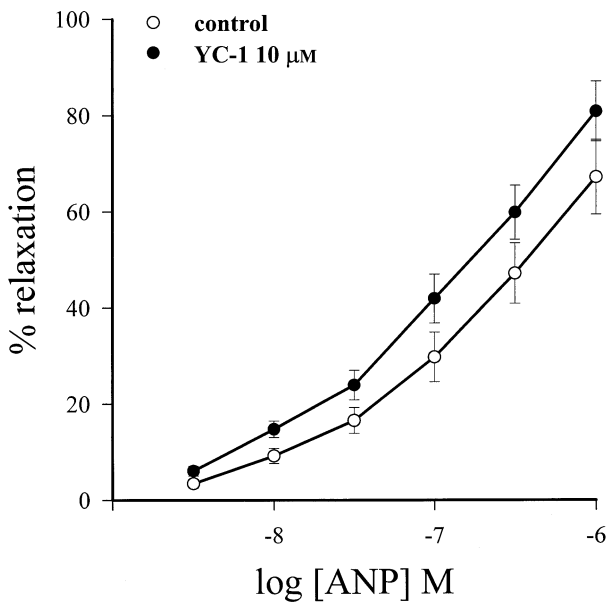

Figure 8 Concentration-response curves showing relaxation to ANP in guinea-pig isolated trachea and the effects of YC-1 (10 μM) on this relaxation. Tissues were treated with indomethacin (3 μM) and propranolol (1 μM) and contracted with 10 μM histamine. Per cent relaxation is given as the mean ± s.e.mean of six experiments.

The effect of YC-1 on phosphodiesterase activity

To investigate the ability of YC-1 to inhibit cycle GMP-hydrolyzing phosphodiesterase activity, we also examined the effect of YC-1 on cyclic GMP breakdown in the cytosol of human platelets. In the presence of YC-1, degradation of cyclic GMP was significantly attenuated; this result is in agreement with the findings of Friebe *et al.* (1998). The IC_{50} value of YC-1 was 31.9 μM ($n=3$). Because the cyclic GMP-binding phosphodiesterase (type V) is the major cyclic GMP-hydrolyzing phosphodiesterase in human platelets, it is likely that the inhibitory effect of YC-1 on cyclic GMP hydrolysis is the result of inhibition of this phosphodiesterase.

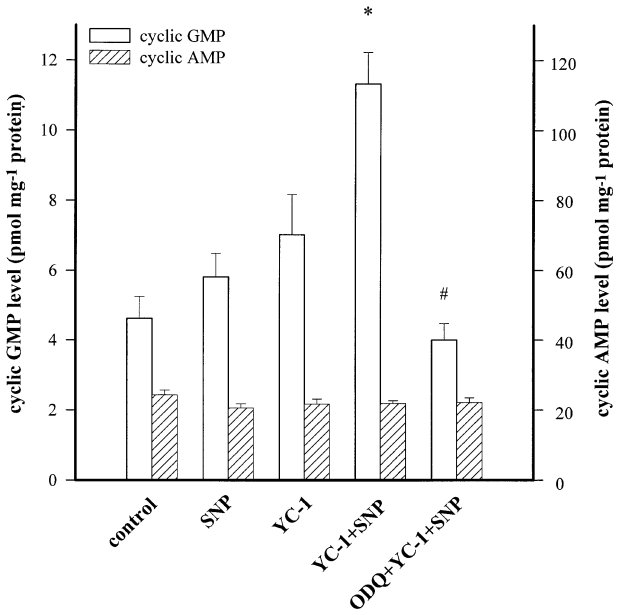

Figure 9 Synergistic effect of YC-1 and SNP on cyclic GMP and cyclic AMP levels in the isolated trachea of guinea-pig. Tissues were incubated with vehicle (control; 0.1% DMSO), SNP (0.3 μM), YC-1 (10 μM), YC-1 (10 μM) plus SNP (0.3 μM) or ODQ (10 μM) followed by YC-1 plus SNP in the presence of IBMX (300 μM). Indomethacin (3 μM) and propranolol (1 μM) were present in this experiment. The values are presented as the mean$\pm$s.e.mean of four experiments. *$P<0.05$ as compared with the control value. #$P<0.05$ as compared with the YC-1 plus SNP.

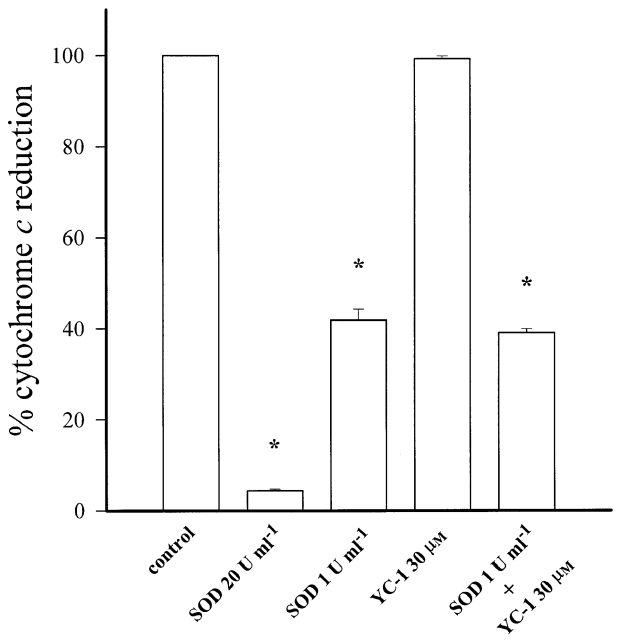

Figure 10 Effects of YC-1 on the reduction of cytochrome *c* by superoxide anion generated from the xanthine/xanthine oxidase system at 25°C. Reduction of cytochrome *c* was measured spectrophotometrically at 550 nm. The values are presented as the mean$\pm$s.e.mean of four experiments. *$P<0.05$ as compared with the control value.

The in vitro *effect of YC-1 on O_2^- generating system*

O_2^- generated by xanthine/xanthine oxidase reduces cytochrome *c*. Compounds capable of scavenging O_2^- can inhibit cytochrome *c* reduction. The initial rate of cytochrome *c* reduction was almost completely inhibited by SOD (20 U ml^{-1}) (from 0.140 ± 0.002 to 0.006 ± 0.001 OD min^{-1}; $n=4$; $P<0.001$). As shown in Figure 10, YC-1 at a concentration up to 30 μM, did not scavenge O_2^- and also had no effect on the removal of O_2^- by SOD (1 U ml^{-1}) in this system.

Discussion

NO has been cited to play an important role in airway function. Several NO-related compounds, such as SNP, and endogenous NO activate sGC, elevate cyclic GMP and relax airway smooth muscle (Katsuki & Murad, 1977; Gruetter *et al.*, 1989). However, there are also indications that NO-donors cause relaxation of tracheal smooth muscle *via* a cyclic GMP-independent mechanism (Zhou & Torphy, 1991; Gaston *et al.*, 1994; Stuart-Smith *et al.*, 1994; Sadeghi-Hashjin *et al.*, 1996a). These successive studies using methylene blue as an inhibitor of sGC to investigate the importance of cyclic GMP in NO-induced bronchodilation led to controversial suggestions. 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 airway smooth muscle. In this study, we demonstrated that tracheal relaxation elicited by the NO-donor SNP or by the NO component of the iNANC system was substantially inhibited by ODQ. These results provide strong evidence that the relaxant responses to exogenously added or endogenously released NO in guinea-pig trachea are completely *via* a cyclic GMP-mediated mechanism, which are in agreement with previous findings (Ellis, 1997; Hwang *et al.*, 1998).

The new substance YC-1, which is a benzylindazole derivative, has been described as an inhibitor of platelet aggregation (Wu *et al.*, 1995) and a dilator of vascular smooth muscle (Mülsch *et al.*, 1997), presumably acting *via* direct, but NO-independent activation of sGC. Moreover, YC-1 sensitizes sGC for its physiological activator NO and also turns CO into a potent activator (Friebe *et al.*, 1996; 1998; Mülsch *et al.*, 1997). These findings have potential pharmacological and physiological implications. However, the activity of YC-1 in tracheal relaxation has not been characterized. Here, we further investigated the effect of YC-1 and its mechanism of action in guinea-pig trachea. YC-1, which elicited only minor effects on relaxation, significantly potentiated the tracheal relaxation caused by NO-donor SNP and by NO component of the iNANC system. This synergistic response of YC-1 was *via* the increasing cyclic GMP levels, since the relaxation caused by exogenous or endogenous NO with YC-1 was inhibited by ODQ. Moreover, YC-1 was without effect on relaxation to the adenylate cyclase activator forskolin or the

non-NO component of iNANC transmitters in guinea-pig trachea.

Similar to the finding obtained with tracheal relaxation a synergism between YC-1 and SNP was observed with increase of cyclic GMP. YC-1 together with SNP, at concentrations that by themselves did not elevate cyclic GMP synthesis, markedly enhanced cyclic GMP but not cyclic AMP levels. The increase of cyclic GMP accumulation was also inhibited by ODQ. It was suggested that the elevation of SNP-induced cyclic GMP formation by YC-1 was due to increasing sensitivity of sGC, because this synergistic effect was in the presence of non-selective phosphodiesterase inhibitor IBMX. Moreover, the relaxation of SNP in the pretreatment of zaprinast, a selective inhibitor of phosphodiesterase type V, was further enhanced by YC-1. This data again suggests a role for potentiation of NO-induced sGC stimulation by YC-1. These results are consistent with the previous reports of YC-1 on purified sGC (Friebe *et al*., 1996; Mülsch *et al*., 1997; Russwurm *et al*., 1998). However, since the ability of YC-1 to inhibit phosophodiesterase type V and the effect of YC-1 to potentiate ANP-induced relaxation, these observations were interpreted as meaning that the potentiation of NO-induced relaxation by YC-1 was at least partially due to inhibition of cyclic GMP breakdown. Similar results have been described by other reporters (Friebe *et al*., 1998; Galle *et al*., 1999). Additionally, considering that O_2^- may inactivate NO/sGC-induced tracheal relaxation (Sadeghi-Hashjin *et al*., 1996b), the effect of YC-1 on the O_2^- generating system was investigated. Our results showed that YC-1 at a concentration up to 30 μM did not scavenge O_2^- and also had no effect on the removal of O_2^- by SOD in a cell-free system. In the present study, it is surprising that although YC-1 could increase the relaxant potency of NO in guinea-pig isolated trachea, it did not affect the tension by itself *via* a cyclic GMP dependent pathway. Definitely, YC-1 can relax rat aortic smooth muscles with an EC_{50} value of about 10–30 μM (Mülsch *et al*., 1997). For the reason that relaxation caused by NO donor SNP was less potent on the guinea-pig trachea (EC_{50} 2.60 μM) than on the rat vascular smooth muscle (EC_{50} 0.04 μM; Mülsch *et al*., 1997); this phenomenon implies that the ineffective relaxation of YC-1 in guinea-pig trachea may be due to less activity of sGC/cyclic GMP in trachea as compared to vascular smooth muscle.

In conclusion, these results indicate that YC-1 potentiates tracheal relaxation to both exogenous and endogenous NO *via* the elevation of cyclic GMP levels. Additionally, YC-1 neither augments responses to activation of adenylate cyclase pathways nor modulates the production of superoxide anions.

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