Mechanism of Carbon Monoxide-Induced Relaxation in the Guinea Pig Ileal Smooth Muscle

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ABSTRACT. The mechanism of carbon monoxide (CO)-induced relaxation were investigated in the guinea-pig ileum. CO (10%) inhibited the 40 mM KCl-induced contraction. This effect was antagonized by ODQ (1 μM), a soluble guanylate cyclase inhibitor. In contrast, CO did not inhibit the 40 mM KCl-induced increase in cytosolic Ca^{2+} level ($[Ca^{2+}]_i$). Cumulative addition of KCl induced a graded increase in both $[Ca^{2+}]_i$ and muscle tension. In the presence of CO, the increase in muscle tension was attenuated whereas the increase in $[Ca^{2+}]_i$ was only slightly decreased. Thus, the $[Ca^{2+}]_i$-tension relationship constructed by cumulative addition of KCl shifted downwards in the presence of CO. Using the patch clamp, CO was found to have little effect on the peak Ba currents (I_{Ba}) when voltage was stepped from –60 mV to 0 mV. From these results, we conclude that CO inhibits contraction of guinea-pig ileum mainly by the decrease in the sensitivity of contractile elements to Ca^{2+} via a cyclic GMP-dependent pathway but not by the inhibition of L-type Ca^{2+} channel.
KEY WORDS: calcium channel, carbon monoxide, guinea-pig, intestinal smooth muscle, relaxation.

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Inhibitory nonadrenergic, noncholinergic (NANC) neurons play an important role in the physiological control of gastrointestinal motility. These neurons are involved not only in gastric receptive relaxation and peristaltic movement throughout the gastrointestinal tract, but also in the relaxation of tonically contracted sphincters [1, 17, 27]. The transmitter(s) involved in the NANC relaxation of gastrointestinal preparations is species- and tissue-dependent and several candidates have been proposed including ATP [4], vasoactive intestinal peptide [14] and nitric oxide (NO) [19, 22].

Recently carbon monoxide (CO) has been suggested to play a role in neuronal transmission in the gastrointestinal tract as an inhibitory NANC transmitter [5, 16, 21]. Like NO, CO is an endogenously produced gas molecule [3, 7, 25, 26]. CO is produced in mammalian tissue via action of heme oxygenase, which is the rate limiting enzyme in degradation of heme. Heme is oxidatively cleaved by heme oxygenase to yield CO and biliverdin [13]. Heme oxygenases has been detected in various type of tissues including smooth muscle [12]. CO has been shown to relax vascular tissues [6, 11], cultured vascular smooth muscle cells [18], certain types of visceral smooth muscles such as ileal smooth muscles from guinea pig [24] and circular smooth muscle strips of feline lower esophageal sphincter [16].

To elucidate the mechanism of CO-induced relaxation in intestinal smooth muscle, we examined the effects of CO on Ca^{2+} channel activity and Ca^{2+} sensitivity of contractile elements.

MATERIALS AND METHODS

Tissue preparations: Male guinea-pigs (300–400 g) were killed by a sharp blow to the neck and exsanguination. A section of ileum was isolated and placed in a physiological salt solution (PSS). Lumen of ileum was cleaned gently with PSS. A 7 mm diameter glass pipette was inserted in the lumen of the ileum and longitudinal muscle layer was separated from the underlying circular muscle layer. Segments of longitudinal muscle layer, about 10 mm long, was used for experiments.

Measurements of muscle tension: Muscle strip was attached to a holder under a resting tension of 0.5 g. After equilibration for 1 hr in PSS, each strip was repeatedly exposed to 40 mM KCl solution until the responses became stable. The high K^+ solution was prepared by replacing NaCl with equimolar KCl. Muscle contraction was recorded isometrically with a force-displacement transducer and recorded with a pen-recorder.

Fura-2 loading and simultaneous measurements of tension and $[Ca^{2+}]_i$: $[Ca^{2+}]_i$ was measured according to the method described by Kwon *et al.* [9] using the fluorescent Ca^{2+} indicator, fura-2. Muscle strips were exposed to the acethoxymethyl ester of fura-2 (fura-2/AM, 5 μM) in the presence of 0.02% cremophor EL for 5–6 hr at room temperature (22–24°C). The muscle strip was illuminated alternatively (48 Hz) with 340 nm and 380 nm light, and the ratio of 500 nm fluorescence induced by 340 nm excitation (F340) and that induced by 380 nm excitation (F380) were detected with a spectrophotometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). Increase in the ratio due to 40 mM KCl was considered as a reference response (100%).

Preparation of cells for patch clamp analysis: The muscle layers of ileum were cut into small pieces and placed in a Ca^{2+}-free PSS. Then, the Ca^{2+}-free PSS was replaced by a

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PSS containing 30 μM Ca^{2+} (low Ca^{2+} PSS) followed by a 30 min incubation at 37°C in a low-Ca^{2+} PSS containing collagenase (0.3 mg/m*l*), papain (0.3 mg/m*l*) and bovine serum albumin (1 mg/m*l*). After the enzyme digestion, tissue fragments were suspended in a PSS containing 120 μM Ca^{2+} and gently agitated. The resulting suspension was centrifuged at $600 \times g$ for 2 min and the cells were resuspended in a PSS containing 0.5 mM Ca^{2+}. Cell suspension was placed on glass cover-slips and stored in a moist atmosphere at 4°C. Experiments were carried out at room temperature (22–24°C).

Whole-cell voltage clamp: Whole-cell membrane current was recorded at room temperature using standard patch-clamp techniques. Patch pipette had a resistance of 3–6 MΩ when filled with a pipette solution. Membrane currents were amplified with an Axopatch 1C voltage-clamp amplifier (Axon Instrument). Command pulses were applied using an IBM-compatible computer and pCLAMP (version 5.5) software. The data were filtered at 2 kHz and displayed on an oscilloscope (Tektronix), a computer monitor and a pen recorder (Universal Oscillograph, Harvard).

Solutions: PSS had following composition (mM): NaCl 126; KCl 6; $CaCl_2$ 2; $MgCl_2$ 1.2; glucose 14; HEPES 10.5 (pH was adjusted to 7.2 with NaOH). PSS was saturated with 100% O_2 at 37°C. Inward currents were isolated by the suppression of K^+ currents using pipette solution containing (mM): CsCl 134, glucose 14, EGTA 0.05, HEPES 10.5 and Na_2ATP 4 (pH was adjusted to 7.2 with CsOH). In the experiments to measure Ca^{2+} currents, $CaCl_2$ in the bath solution was replaced by $BaCl_2$.

Preparation of CO solution: CO was applied by superfusing the preparations with CO-containing PSS which was prepared by bubbling PSS in a container of about 100 m*l* volume with a gas mixture of 10 vol. % CO and 90 vol. % O_2. The resulting 10% CO-containing PSS was applied by changing the solution in the recording chamber. The estimated CO concentration at 10% CO-containing PSS was about 100 μM. Different volumes of CO and O_2 were mixed to achieve the desired concentrations. In a preliminary experiments, it was confirmed that different volumes of deoxygenated PSS that served as control produced no significant effect on both the resting tone and the 40 mM KCl-induced contraction of the guinea pig ileum.

Statistics: Results of the experiments are expressed as mean ± S.E.M. Unpaired Student's *t*-test was used for statistical analysis of the results and the number of preparations taken from separate animals was indicated by n. P values less than 0.05 were considered to be significantly different.

RESULTS

CO caused a concentration-dependent relaxation in the 40 mM KCl-induced contraction, and the maximal relaxation was achieved at 10% (Table 1). CO (10%) had no effect on the basal tension of the guinea-pig ileal muscle strips. After CO was applied to the tissues precontracted with 40 mM KCl, a sustained relaxation was induced (Fig. 1A). In the

Table 1. Effects of O_2, N_2 and CO on 40 mM KCl-induced contraction in guinea-pig ileum

Condition	Relaxation (%)	n
O_2-100%, KCl-40 mM	0	8
O_2-95%, CO-5%, KCl-40 mM	11.4 ± 3.3*	7
O_2-95%, N_2-5%, KCl-40 mM	1.2 ± 0.8	5
O_2-90%, CO-10%, KCl-40 mM	36.4 ± 6.7*	5
O_2-90%, N_2-10%, KCl-40 mM	4.5 ± 3.4	5
O_2-80%, CO-20%, KCl-40 mM	43.2 ± 7.1*	5
O_2-80%, N_2-20%, KCl-40 mM	15.3 ± 4.3	5

Muscle tension are expressed by relative values taking the value in the resting ileum as 0% and the high K^+-stimulated ileum as 100%. Each value represents mean ± S.E.M. n, number of experiments.
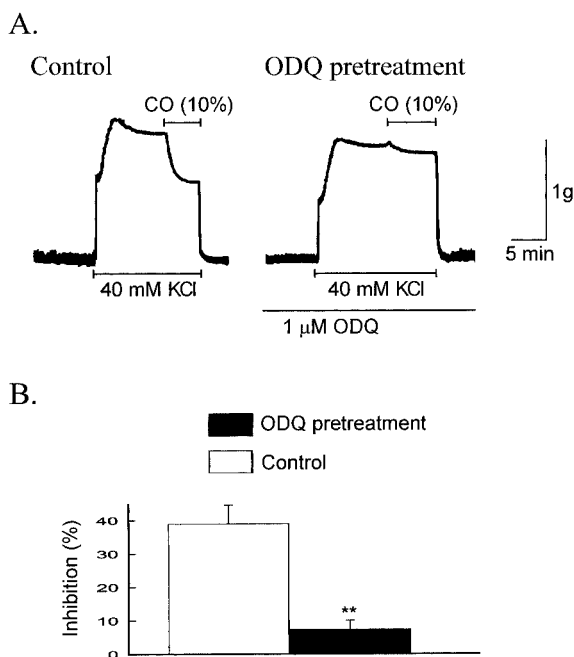* $p < 0.05$ compared with the corresponding control under same concentrations of N_2.

Fig. 1. The inhibitory effect of ODQ (1 μM), a soluble guanylate cyclase inhibitor, on the CO-induced relaxation in guinea-pig ileum. (A) Record of isometric contraction showing that ODQ suppressed the CO (10%)-induced relaxation of longitudinal strip precontracted with 40 mM KCl. When the muscle tension induced by 40 mM KCl reached a steady state level, CO (10%) was added. (B) Summary of the effect of ODQ on the relaxation induced by CO (10%) (n=6). The tonic contractions of intestinal tissues induced by KCl before and after the application of CO were represented by open and solid columns, respectively. ** $p < 0.01$.

presence of CO, the 40 mM KCl-induced contraction was inhibited by 39.8 ± 5.8% (n=6).

To know if the CO-induced relaxation is mediated by the activation of a cyclic GMP signal transduction pathway, the effect of ODQ, a soluble guanylate cyclase inhibitor, was examined. ODQ (1 μM) had no effect on either the resting
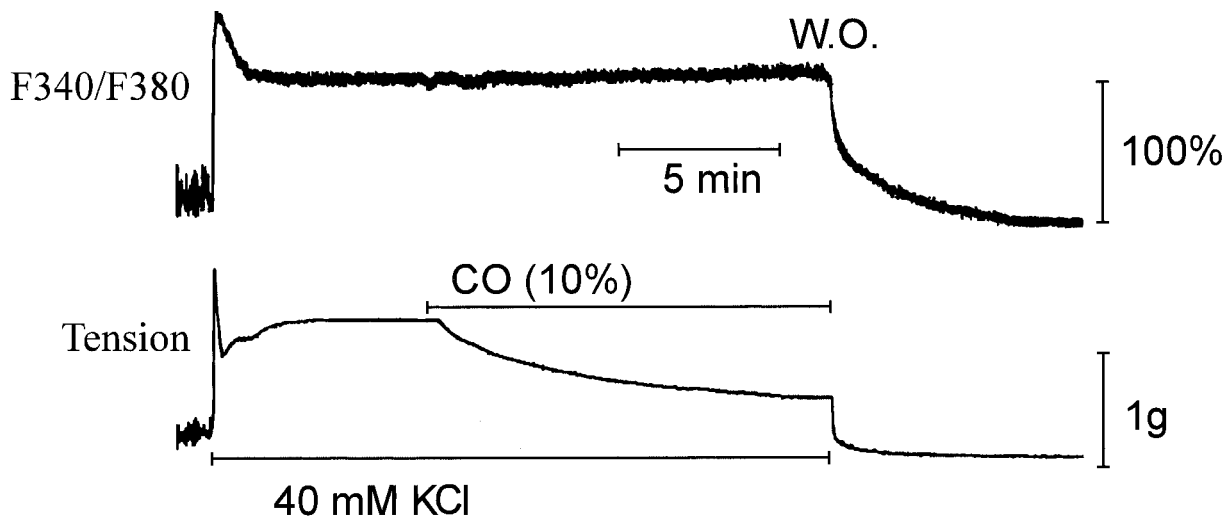
Fig. 2.   The effect of CO on KCl-stimulated $[Ca^{2+}]_i$ (upper trace) and muscle tension (lower trace) in the guinea-pig ileum. 100% represents the 40 mM KCl-induced sustained increase in $[Ca^{2+}]_i$ before the addition of CO. When $[Ca^{2+}]_i$ and muscle tension induced by 40 mM KCl reached a steady state level, CO was added.

tension level or the KCl-induced tonic contraction (Fig. 1A). In the tissues pretreated with ODQ for 20 min, however, the CO-induced relaxation of the muscle precontracted with KCl (40 mM) was reduced significantly from $39.8 \pm 5.8\%$ to $5.2 \pm 2.1\%$ (p<0.05, n=6) (Figs. 1A and 1B).

To examine whether the inhibitory effect of ODQ on the 40 mM KCl-induced contraction was due to the blockade of the cyclic GMP pathway, the effect of R_P-8-Br-PET-cGMP, the protein kinase G inhibitor at 10 μM as well as the interaction of R_P-8-Br-PET-cGMP with ODQ were further studied.   This R_P-8-Br-PET-cGMP pretreatment significantly reduced the CO-induced relaxation from 40.4 ± 6.6 to 15.1 ± 4.2 (n=6).   The inhibitory effect of ODQ on the CO-induced relaxation was not potentiated by R_P-8-Br-PET-cGMP.

As shown in Fig. 2, high K^+ (40 mM) induced sustained increases in the muscle tension and $[Ca^{2+}]_i$ in the muscle loaded with a Ca^{2+} indicator, fura-2.   Approximately 5 min after the application of the KCl, the levels of muscle tension and $[Ca^{2+}]_i$ reached a plateau.   In the presence of KCl, addition of CO inhibited the muscle tension to $43.3 \pm 7.3\%$ (n=5) without changing the plateau $[Ca^{2+}]_i$.   The effect of CO was abolished by washout (not shown).

Cumulative addition of KCl induced concentration-dependent increase in both muscle tension and $[Ca^{2+}]_i$ (Fig. 3) and there is a positive correlation between $[Ca^{2+}]_i$ and muscle tension.   CO inhibited the 40 mM KCl-stimulated muscle tension without changing $[Ca^{2+}]_i$, resulting in the downward shift of the $[Ca^{2+}]_i$-tension relationship.

The effects of CO on Ca^{2+} currents through the voltage-dependent Ca^{2+} channels were examined in single ileal smooth muscle cells (Fig. 4A).   When Ba^{2+} was used instead of Ca^{2+} as the charge carrier, Ba^{2+} currents (I_{Ba}) was elicited by depolarization from a holding potential of -60 mV to a

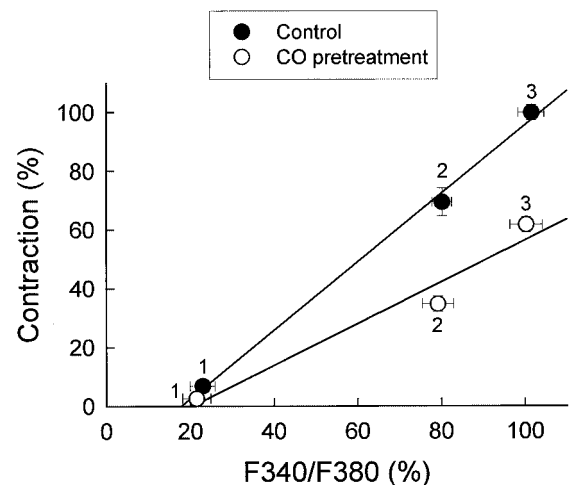Fig. 3.   Effect of cumulative application of KCl on $[Ca^{2+}]_i$-tension relationship in the absence and presence of CO. 100% represents the 40 mM KCl-induced increases in $[Ca^{2+}]_i$ and muscle tension measured before the cumulative addition of KCl. CO was added 5 min before the addition of KCl. The concentration of KCl is indicated by numbers (1, 15 mM; 2, 25 mM; 3, 40 mM). Each point represents the mean of 6 to 8 experiments. S.E.M. is shown by vertical and horizontal bars. The regression line was drawn by least-square method.

test potential of 0 mV for 80 ms at 0.1 Hz.   Application of CO showed no effect on the peak I_{Ba} (Figs. 4A and 4B).

DISCUSSION

We have shown in the guinea-pig ileum that exogenous CO reversibly inhibited the contraction induced by high K^+-
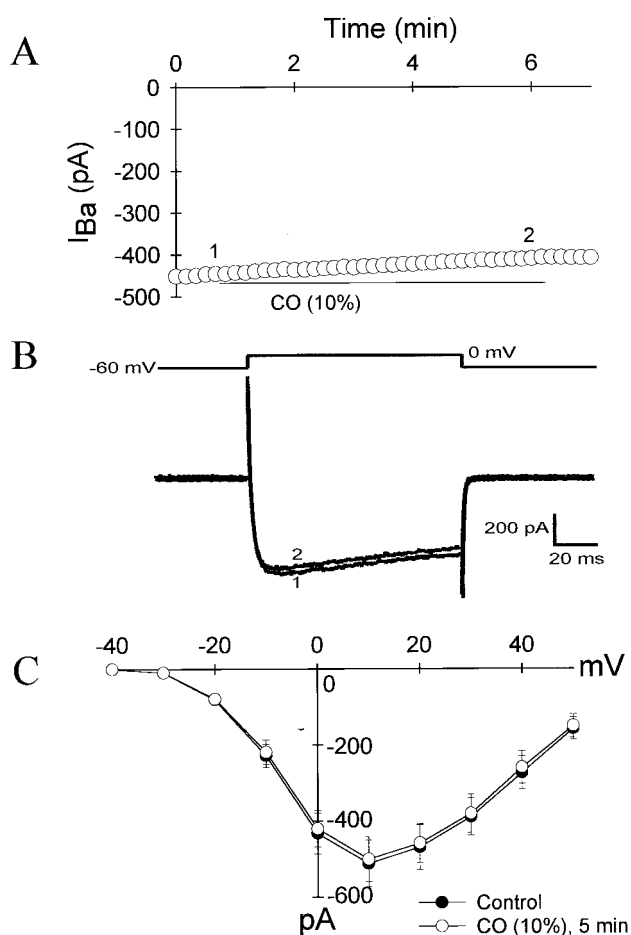
A

B

C

Fig. 4.   Effect of CO on Ba currents (I_{Ba}) in ileal smooth muscle cells. I_{Ba} was elicited by 80 ms depolarization from –60 mV to 0 mV at 0.1 Hz. (A) Time course of the peak I_{Ba} elicited by depolarizing pulses to 0 mV from a holding potential of –60 mV every 10 s under control (1) and in presence of CO (10%) (2). (B) Representative tracing of I_{Ba} from the two time-points labelled in (A). (C) Current-voltage relationships for the peak I_{Ba}. Control and in the presence of CO. I_{Ba} was activated by incremental 10 mV depolarizing steps from a holding potential of -60 mV to + 50

depolarization (Fig. 1).   We have also shown that CO-induced relaxation was inhibited by ODQ, a selective inhibitor of soluble guanylate cyclase. R_P-8-Br-PET-cGMP at a submaximal dose, also reduced significantly the CO-induced relaxation.   It has been known that actions of cyclic GMP are 1) to activate the K^+-channels to inhibit the voltage-operated Ca^{2+}-channels [1, 2, 15], 2) to directly inhibit the voltage-operated Ca^{2+} channels [10], 3) to activate the Ca^{2+} pump on the plasma membrane [20], and 4) to decrease the sensitivity of contractile elements to Ca^{2+} [8].   CO is considered to produce effects on smooth muscle by an activation of soluble guanylate cyclase, resulting in an increase in intracellular cyclic GMP levels, and relaxation [6, 11].

This seems to be the case also in the guinea-pig ileum, where an increase of cyclic GMP concentrations after CO exposure, and an inhibiting effect of methylene blue on the CO-induced relaxation has been demonstrated [24]. R_P-8-Br-PET-cGMP is a membrane-permeable and specific inhibitor of cyclic GMP-dependent protein kinase [5]. These data indicate that the mechanism of CO-induced relaxation of guinea-pig ileum was primarily through the activation of the guanylate cyclase/ cyclic GMP system.

The contraction of smooth muscle is regulated not only by $[Ca^{2+}]_i$ but also by Ca^{2+}-sensitivity of the contractile apparatus, which is also regulated by the intracellular signaling systems.   It is well known that NO-mediated relaxation of smooth muscles involves elevated cGMP and cGMP inhibit the Ca^{2+}-induced contraction by the decrease in Ca^{2+} sensitivity of contractile elements [9]. Like NO, CO is also known to increase intracellular cGMP levels, this suggests that CO may also affect the Ca^{2+} sensitivity. In the present study, cumulative addition of KCl induced a graded increase in $[Ca^{2+}]_i$ and muscle tension. In the presence of CO, cumulative addition of KCl induced smaller contraction than in the absence of CO. On the other hand, the increase in $[Ca^{2+}]_i$ induced by cumulative addition of KCl was only slightly decreased in the presence of CO, and the $[Ca^{2+}]_i$-tension relationship shifted downwards (Fig. 3). Since CO shifted the $[Ca^{2+}]_i$-tension relationship to downwards, inhibition of high K^+-induced contraction by CO may be attributable to the decrease in the sensitivity contractile elements to Ca^2.

In vascular smooth muscle, Lin and McGrath [11] reported that CO-induced relaxation is due to decrease in Ca^{2+} concentration. In our experiments with ileal muscle, however, CO decreased neither $[Ca^{2+}]_i$ (Fig. 2) nor the inward Ca^{2+} current (Fig. 4). These findings suggest that the inhibition of L-type Ca^{2+} currents may not be responsible for the CO-induced relaxation in the guinea-pig ileal smooth muscle and that there is a species- and/or tissue-difference in the effects of CO.

In a variety of smooth muscles, hypoxia or metabolic inhibition resulted in a significant reduction in force with no change or increases in $[Ca^{2+}]_i$ either at rest or in activated tissues [23]. In the present experiments, we confirmed that resting tone of the ileum was not inhibited by CO. Furthermore, the fact that both ODQ and R_P-8-Br-PET-cGMP inhibited the relaxant effect of CO suggest that the effect of CO is mediated by cyclic GMP but not by metabolic inhibition. However, further investigation is needed to draw conclusion on the possible involvement of metabolic inhibition in the CO-induced relaxation.

In summary, we demonstrated that CO, through the cyclic GMP-dependent signaling pathways, inhibits smooth muscle contraction by decreasing Ca^{2+} sensitivity of contractile elements in the guinea-pig ileal smooth muscle.

REFERENCES

1. Abrahamsson, H. 1986. Non-adrenergic non-cholinergic nervous control of gastrointestinal motility patterns. *Arch Int. Pharmacodyn.* 280: 50–61.

2. Archer, S.L., Huang, J.M.C., Hampl, V., Nelson, D.P., Shultz, P.J. and Weir, E.K. 1994. Nitric oxide and cyclic GMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cyclic GMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* 91: 7583–7587.

3. Burne, B. and Ullrich, V. 1987. Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanyl cyclase. *Mol. Pharmacol.* 32: 497–504.

4. Burnstock, G. 1990. Overview. Purinergic mechanisms. *Ann. N. Y. Acad. Sci.* 603: 1–17.

5. Chakder, S., Cao, G.-Y., Lynn, R.B. and Rattan, S. 2000. Heme oxygenase activity in the internal anal sphincter: Effects of nonadrenergic, noncholinergic nerve stimulation. *Gastroenterology* 118: 477–486.

6. Graser, T., Vedernikov, Y.P. and Li, D.S. 1990. Study on the mechanism of carbon monoxide induced endothelium-independent relaxation in porcine coronary artery and vein. *Biomed. Biochim. Acta* 49: 293–296.

7. Ingi, T., Cheng, J. and Ronnett, G.V. 1996. Carbon monoxide: an endogenous modulator of the nitric oxide-cyclic GMP signaling system. *Neuron* 16: 835–842.

8. Karaki, H., Ozaki, H., Hori, M., Mitsui-Saito, M., Amano, K., Harada, K., Miyamoto, S., Nakazawa, H., Won, K.J. and Sato, K. 1997. Calcium movements, distribution, and functions in smooth muscle. *Pharmacol. Rev.* 49: 157–230.

9. Kwon, S.C., Park, K.Y., Ahn, D.S., Lee, H.Y. and Kang, B.S. 2000. The effect of NO donor on contraction, cytosolic Ca^{2+} level and ionic currents in guinea-pig ileal smooth muscle. *Korean J. Physiol. Pharmacol.* 4: 33–40.

10. Lorenz, J.N., Bielefeld, D.R. and Sperelakis, N. 1994. Regulation of calcium channel current in A7r5 vascular smooth muscle cells by cyclic nucleotides. *Am. J. Physiol.* 266: C1656–C1663.

11. Lin, H. and McGrath, J.J. 1988. Carbon monoxide effects on calcium levels in vascular smooth muscle. *Life. Sci.* 43: 1813–1816.

12. Maines, M.D. 1988. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J.* 2: 2557–2568.

13. Maines, M.D. 1993. Carbon monoxide: an emerging regulator of cyclic GMP in the brain. *Mol. Cell. Neurosci.* 4: 389–397.

14. Makhlouf, G.M. and Grider, J.R. 1993. Nonadrenergic noncholinergic inhibitory transmitters of the gut. *News Physiol. Sci.* 8: 195–199.

15. Naseem, K.M., Mumtaz, F.H., Thomson, C.S., Sullivan, M.E., Khan, M.A., Morgan, R.J., Mikhailidis, D.P. and Bruckdorfer, K.R. 2000. Relaxation of rabbit lower urinary tract smooth muscle by nitric oxide and carbon monoxide: modulation by hydrogen peroxide. *Eur. J. Pharmacol.* 387: 329–335.

16. Ny, L., Grunemar, L., Larsson, B., Ekstrom, P. and Andersson, K.E. 1995. Carbon monoxide as a putative messenger molecule in the feline lower esophageal sphincter of the cat. *Neuroreport* 6: 1261–1265.

17. Otterson, M.F. and Sarr, M.G. 1993. Normal physiology of small intestinal motility. *Surg. Clin. North Am.* 73: 1172–1192.

18. Ramos, K.S., Lin, H. and McGrath, J.J. 1989. Modulation of cyclic guanosine monophosphate levels in cultured aortic smooth muscle cells by carbon monoxide. *Biochem. Pharmacol.* 38: 1368–1370.

19. Rand, M.J. and Li, C.G. 1995. Nitric oxide as a neurotransmitter in peripheral nerves: nature of transmitter and mechanism of transmission. *Annu. Rev. Physiol.* 57: 659–682.

20. Rashatwar, S.S., Cornwell, T.L. and Lincoln, T.M. Effects of 8-bromo-cyclic GMP on Ca^{2+} levels in vascular smooth muscle cell: Possible regulation of Ca^{2+}-ATPase by cyclic GMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* 84: 5685–5689.

21. Rattan, S. and Chakder, S. 1993. Inhibitory effect of CO on internal anal sphincter: heme oxygenase inhibitors inhibit NANC relaxation. *Am. J. Physiol.* 265: G799–G804.

22. Sanders, K.M. and Ward, S.M. 1992. Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.* 262: G378–G392.

23. Taggart, M.J. and Wray, S. 1998. Hypoxia and smooth muscle function: key regulatory events during metabolic stress. *J. Physiol.* 509: 315–325.

24. Utz, J. and Ullrich, V. 1991. Carbon monoxide relaxes ileal smooth muscle through activation of guanylate cyclase. *Biochem. J.* 41: 1195–1201.

25. Verma, A., Hirsch, D., Glatt, C., Ronnette, G. and Snyder, S. 1993. Carbon monoxide: a putative neural messenger. *Science* 259: 381–384.

26. Wang, R. 1998. Resurgence of carbon monoxide: an endogenous gaseous vasorelaxing factor. *Can. J. Physiol. Pharmacol.* 76: 1–5.

27. Zenilman, M.E. 1993. Origin and control of gastrointestinal motility. *Surg. Clin. North Am.* 73: 1081–1099.