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The Effect of Carbon Monoxide on Contraction, Cytosolic Ca^{2+} Level and Ionic Currents in Guinea Pig Ileal Smooth Muscle

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The aim of this study was to clarify the mechanism of the inhibitory action of carbon monoxide (CO) on contraction, by measuring cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) and ionic currents in guinea-pig ileum. CO (10%) inhibited 40 mM KCl-induced contraction and this effect was blocked by ODQ (1 μM), a soluble guanylyl cyclase (sGC) inhibitor. CO inhibited the 40 mM KCl-induced contraction without changing $[Ca^{2+}]_i$. 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. Using the patch clamp technique with a holding potential of −60 mV, we found that CO had little effect on the peak Ba currents (I_{Ba}) when voltage was stepped from −60 mV to 0 mV. In addition, CO showed no effect on the depolarization-activated outward K^+ currents in the all potential ranges. We conclude that CO inhibits smooth muscle contraction mainly by decreasing the Ca^{2+} sensitivity of contractile elements via a cGMP-dependent pathway, not by involving L-type Ca^{2+} and outward-potassium currents in guinea-pig ileum.

Key Words: Carbon monoxide, Intestinal smooth muscle relaxation, cGMP

INTRODUCTION

Inhibitory nonadrenergic noncholinergic (NANC) neurons play an important role in the physiological control of gastrointestinal motility. They are involved in gastric receptive relaxation, in peristaltic movement throughout the gastrointestinal tract and in relaxation of tonically contracted sphincters (Abrahamsson, 1986; Otterson & Sarr, 1993; Zenilman, 1993). The nature of the transmitter(s) involved in NANC relaxation of gastrointestinal preparations is species- and tissue-dependent and several candidates have been proposed; vasoactive intestinal peptide (VIP; Makhlouf & Grider, 1993) and nitric oxide (NO; Rand & Li, 1995; Sanders & Ward, 1992). In rat gastric fundus, although the blockade of both the inhibitory neurotransmitters, VIP and NO, caused an additive and significant blockade of the smooth muscle relaxation in response to NANC nerve stimulation, a part of the NANC mediated relaxation was still observed (D'Amato et al, 1992). The nature of the unknown alternative neurotransmitter(s) remains elusive.

Recently carbon monoxide (CO) has been suggested to have a role in neuronal transmission and was also proposed as an inhibitory NANC transmitter in the gastrointestinal tract (Rattan & Chakder, 1993; Ny et al, 1995; Chakder et al, 2000). It is well known that CO is produced in mammalian tissue via action of heme oxygenase (HO), which is the rate limiting enzyme in degradation of heme. Heme is oxidatively cleaved by HO to yield CO and biliverdin (Maines, 1993). Heme oxygenases have been detected in many tissues that have been tested including smooth muscle cells (Maines, 1988). And CO induces relaxations of vascular tissues (Lin & McGarth, 1988; Graser et al, 1990), cultured vascular smooth muscle cells (Ramos et al, 1989), and also relaxes certain types of visceral smooth muscles such as ileal smooth muscles from guinea pig (Utz & Ullrich, 1988) and isolated circular

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smooth muscle strips of feline lower esophageal sphincter (Ny et al, 1995). CO activates endogenous soluble guanylyl cyclase resulting in an increased production of guanosine 3',5'-cyclic monophosphate (cGMP). cGMP-dependent protein phosphorylation is found in several tissues including smooth muscle (Casnellie & Greengard, 1974). cGMP can regulate ion channels either by acting directly on channels, as in nonselective cation channels in the retina (Fesenko et al, 1981), or by acting indirectly as in activation of potasium channels in the rabbit cornea (Farrugia & Rae, 1992) and canine jejunum (Farrugia et al, 1998). Zinc protoporphyrin IX has been shown to be a selective inhibitor of HO (Maines, 1981). Based on the activity HO and the HO inhibitor, recent studies suggested a role of CO as a messenger molecule similar to NO (Verma et al, 1993). The action of CO on the gastrointestinal smooth muscle and its role are not known.

Based on these findings, we now assume that CO induces relaxation mainly by suppressing cytosolic Ca^{2+} concentration in smooth muscle cells. In order to elucidate the precise mechanisms of the relaxant effect of CO on intestinal smooth muscle, we examined its effect on $[Ca^{2+}]_i$ and muscle tension in intact guinea-pig ileum. We also used whole-cell voltage clamp technique in freshly dispersed ileal smooth muscle cells and determined the effects of CO on the ionic currents, and also analyzed whether Ca^{2+} sensitivity was changed by CO as well.

METHODS

Tissue preparations

Male guinea-pigs (300~400 g) were killed by a sharp blow to the neck and exsanguination. The abdomen was opened and a section of ileum was isolated promptly and placed in 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 were used for experiments.

Measurements of muscle tension

Each strip was attached to a holder under a resting tension of 0.5 g. After equilibration for 1 h in a 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. These solution were saturated with 100% O_2 at 37°C to maintain pH at 7.2. Muscle contraction was recorded isometrically with a force-displacement transducer and recorded on 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 (2000) 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). After loading, the muscle strips were washed with PSS at 37°C for 20 min to remove uncleaved fura-2/AM and were held horizontally in a temperature-controlled chamber. One end of the muscle strip was connected to a force-displacement transducer to monitor the muscle contraction. The muscle strip was illuminated alternatively (48 Hz) with 340 nm and 380 nm lights. Then 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 Ca^{2+}-free PSS. The Ca^{2+}-free PSS was replaced by PSS containing 30 μM Ca^{2+} (low Ca^{2+} PSS). 30 min incubation at 37°C was carried out in fresh low Ca^{2+} PSS containing collagenase (0.3 mg/ml), papain (0.3 mg/ml) and bovine serum albumin (1 mg/ml). After this enzyme digestion, tissue fragments were suspended in a fresh 120 μM Ca^{2+}-containing PSS and gently agitated. The resulting suspension was centrifuged at 600×g for 2 min and the cells were resuspended in a 0.5 mM Ca^{2+}-containing PSS. 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 currents were 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 measured 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 a oscilloscope (Tektronix), a computer monitor and a pen recorder (Universal Oscillograph, Harvard).

Solutions

PSS used as the bath solution had following composition (mM): NaCl 126; KCl 6; $CaCl_2$ 2; $MgCl_2$ 1.2; glucose 14; HEPES 10.5 (pH 7.2 with NaOH). The patch pipette solution for outward K^+ currents had the following composition (mM): KCl 134; $MgCl_2$ 1,2; Na_2ATP 1; GTP 0.1; EGTA 0.05, glucose 14; HEPES 10.5 (pH 7.2 with KOH). Inward currents were isolated by the suppression of K^+ currents using pipette solution contained (mM): filled with CsCl 134, glucose 14, EGTA 0.05, HEPES 10.5 and Na_2ATP 4 (pH 7.2 with CsOH). In experiments dealing with 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 ml 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. In a preliminary experiment, it was confirmed that different volumes of deoxygenated PSS that served as control produced no significant effect on the resting tone of the guinea pig ileum.

Statistics

The results of the experiments are expressed as mean±S.E.M. and n values refer to the number of strips of ileum. Paired Student's t-test was used for statistical analysis of the results and $p<0.05$ was considered to be significantly different.

RESULTS

Effects of CO on muscle-contraction

The effects of CO (10%) on high K^+ (40 mM)-induced contraction were examined. CO had no effect on the basal tension of the guinea-pig ileal muscle strips. After CO was applied to the tissues, which were precontracted with 40 mM KCl, a sustained relaxation occurred (Fig. 1A). In the presence of CO, the 40 mM KCl-induced tissue contraction was inhibited by 39.8±5.8% (n=6). To ascertain that the CO-induced relaxation may be mediated by the activation of a cyclic GMP signal transduction pathway, a soluble guanylate cyclase inhibitor ODQ was used. ODQ (1 μM) had no effect on either the resting

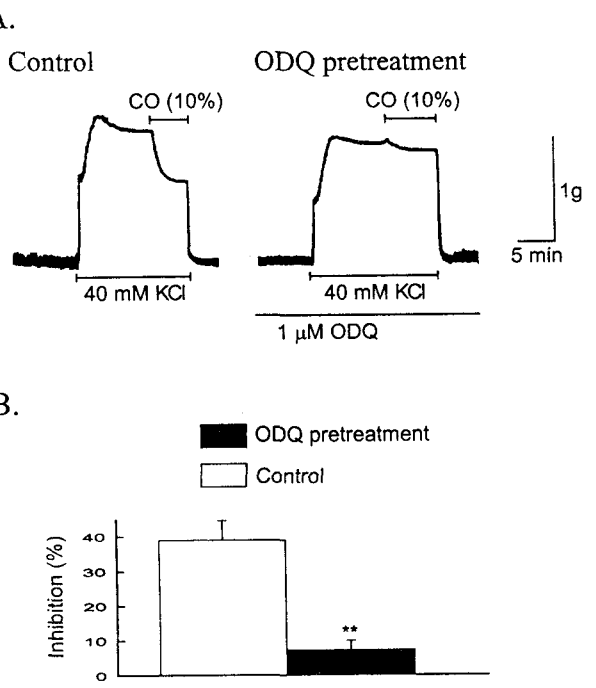

Fig. 1. The inhibitory effect of ODQ (1 μM), a soluble guanylyl cyclase inhibitor, on the CO-induced relaxation in guinea-pig ileum. (A) Actual isometric contraction record showing that ODQ suppressed the CO (10%)-induced relaxation of longitudinal strip precontracted with 40 mM KCl. When the muscle 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. **$p<0.01$.

tension level or the 40 mM 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±5.8% to 5.2±2.1% ($p<0.05$, n=6) (Fig. 1A & 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-cyclic GMPS, the protein kinase G inhibitor, at 30 μM as well as the interaction of R_P-8-Br-cyclic GMPS with ODQ were further studied. This R_P-8-Br-cyclic GMPS pretreatment significantly reduced the CO-induced relaxation from 41.4±6.7% to 17.8±5.1% ($p<0.05$, n=6). The inhibitory effect of ODQ on the CO-induced relaxation was not potentiated by R_P-8-Br-cyclic GMPS (30 μM) (data not shown).

Effects of CO on the muscle-contraction and $[Ca^{2+}]_i$

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 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±7.3% (n=5) without changing the plateau $[Ca^{2+}]_i$. The effects of CO was removed by washout (not shown).

Cumulative addition of KCl induced concentration-dependent increases in both muscle tension and $[Ca^{2+}]_i$ (Fig. 3). There is a positive correlation between $[Ca^{2+}]_i$ and muscle tension. CO inhibited the 40 mM KCl-stimulated muscle tension without changing $[Ca^{2+}]_i$. So, the slope of $[Ca^{2+}]_i$-tension curve shifted downward. This result suggests that CO inhibited the high K^+-induced contraction mainly by the decrease in Ca^{2+} sensitivity of contractile elements.

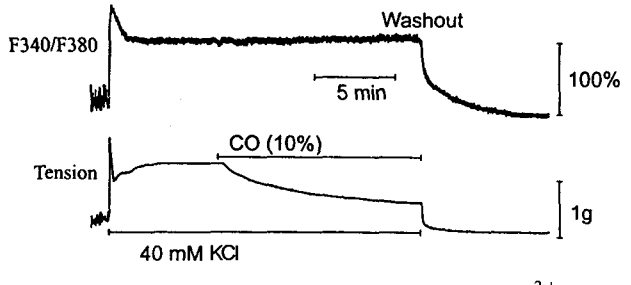

Fig. 2. The effect of CO on KCl-stimulated $[Ca^{2+}]_i$ (upper trace) and muscle tension (lower trace) in guinea-pig ileum. 100% represents the 40 mM KCl-induced sustained increase in $[Ca^{2+}]_i$ before addition of CO. When $[Ca^{2+}]_i$ and muscle tension induced by 40 mM KCl reached a steady state level, CO was added.

Effects of CO on the inward Ca^{2+} currents

The effects of CO on Ca^{2+} currents through voltage-dependent Ca^{2+} channels were examined in single ileal smoth muscle cells in a solution containing 2 mM Ba^{2+} (Fig. 4A). When Ba^{2+} was used instead of Ca^{2+} as the charge carrier, Ba^{2+} currents (I_{Ba}) were elicited by depolarization from a holding potential of −60 mV to a test potential of 0 mV for 80 ms at 0.1 Hz. Application of CO showed no effect on peak I_{Ba} itself (Fig. 4A & 4B).

Effects of CO on the outward K^+ currents

Cells dialyzed with high K^+ solution showed large outward current during 900-ms depolarizing steps from a holding potential of −60 mV to potentials positive to +50 mV. To determine whether the K^+

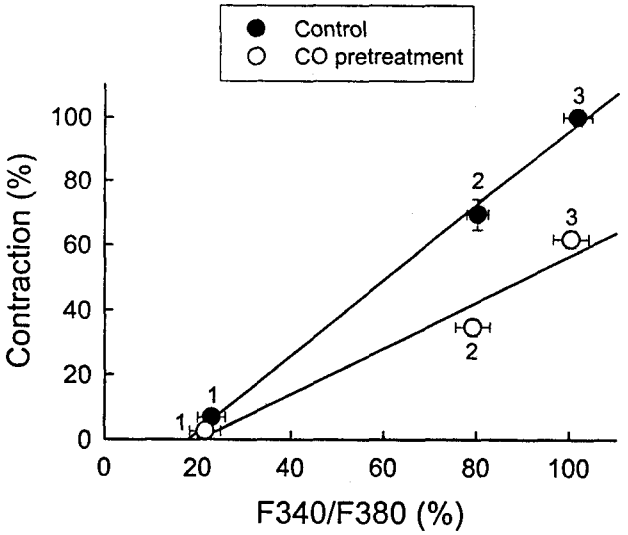

Fig. 3. Effect of cumulative application of KCl on $[Ca^{2+}]_i$-tension relationship in the absence (●) and presence of (○) CO. 100% represents the 40 mM KCl-induced increases in $[Ca^{2+}]_i$ and muscle tension measured before the cumulative addition of KCl. CO was added 5 min before the addition of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

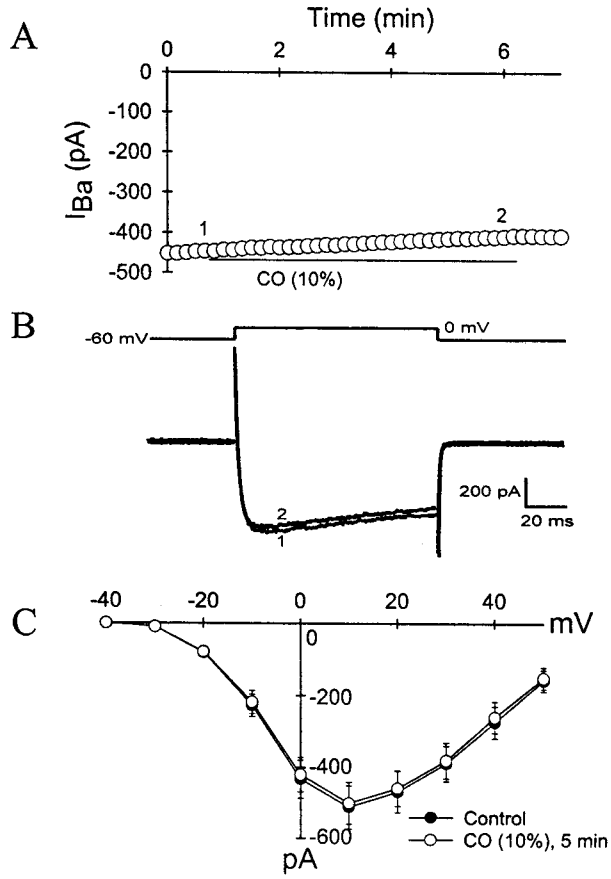

Fig. 4. Effect of CO on Ba currents (I_{Ba}) in ileal smooth muscle cells. I_{Ba} was elicited by 80 ms depolarization from −60 mV to 0 mV at 0.1 Hz. (A) Time course of the peak I_{Ba} elicited by depolarizing pulses to 0 mV from a holding potential of −60 mV every 10 s under control (1) and in presence 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 mV.

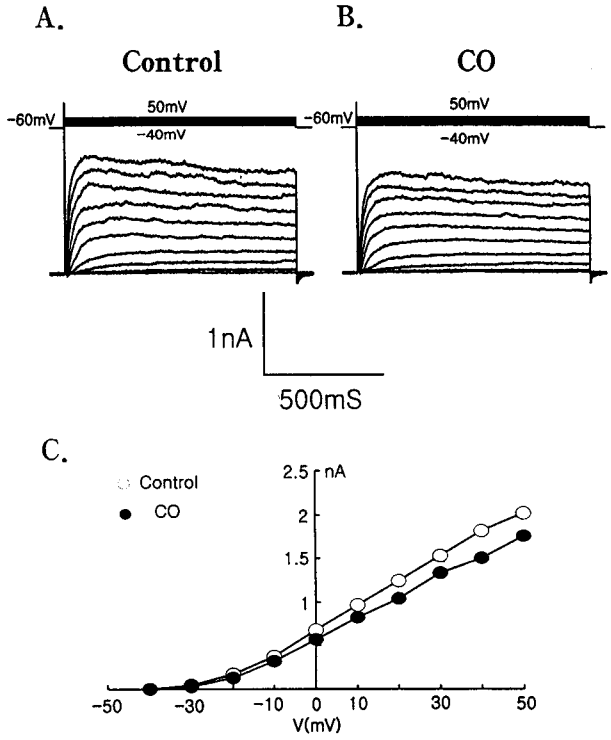

Fig. 5. The effect of CO on the outward K^+ currents recorded from ileal smooth muscle cells in the absence (A) or presence (B) of CO (10%). In each panel the cell was held at −60 mV, and test depolarization with durations of 900 ms were applied from −40 mV to +50 mV in 10-mV increments. Currents are recorded using a pipette containing 0.05 mM EGTA. (A): Whole cell currents under control conditions. (B): Effect of 10% CO on the whole cell currents. (C): Current-voltage relationships recorded when maximum effects were seen at 5 min after addition to bath. ●: Control, ○: 10% CO.

current is activated by CO, the amplitude of the whole cell K^+ current was measured before and after exposure to CO. CO (10%) had little effect on the net outward current (Fig. 5A & 5B). Maximum effects were seen at 5 min after administration. The current amplitude at the last step voltage (from −60 mV to +50 mV) slightly decreased from 1332±189 to 1183±203 pA (n=5) with the addition of CO to the bath. Application of CO showed no effect on the current-voltage relationship (Fig. 5C).

DISCUSSION

We have shown in guinea-pig ileum that exogenous CO inhibited the contraction induced by stimulation with high K^+-deploarization. We have also shown that CO-induced relaxation was inhibited by ODQ which demonstrated that CO-mediated relaxation was mediate by sGC activation. R_P-8-Br-cyclic GMPS 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 (Abrahamsson, 1986; Archer et al, 1994), 2) to directly inhibit the voltage-operated Ca^{2+} channels (Lorenz et al, 1994), 3) to activate the Ca^{2+} pump on the plasma membrane (Rashatwar et al, 1987), and 4) to decrease the sensitivity of

contractile elements to Ca^{2+} (Karaki et al, 1997). Because the effect of CO can be antagonized by ODQ (Fig. 1), it is likely that this mechanism is also mediated by sGC system coupled to CO. CO has been reported to exert the effect by activating sGC in the target cell, leading to an increase in intracellular cGMP (Burne & Ullrich, 1987; Verma et al, 1993). 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 present results showed that CO reversibly inhibited the 40 mM KCl-induced contraction (Fig. 1). Because the effect of CO can be antagonized by ODQ, it is likely that this mechanism is also mediated by sGC system coupled to CO. CO has been reported to exert the effect by activating sGC in the target cell, leading to an increase in intracellular cGMP (Burne et al, 1987; Verma et al, 1993). These data indicate that the mechanism of CO-induced relaxation of guinea-pig ileum was primarily through the activation of sGC.

Numerous studies in smooth muscle have shown that CO mediate relaxation in part by decreasing $[Ca^{2+}]_i$ (Lin & McGrath, 1988; Trishmann et al, 1991). The effect of CO has been hypothesized as a consequence of the CO-induced decrease in intracellular free calcium concentration. In this context, voltage-dependent (L-type) Ca^{2+} channels could be possible targets for the action of CO. It appears that L-type Ca^{2+} channels are known to be modulated by several intracellular second-messenger systems, including cGMP-dependent protein kinase pathway. Lin & McGrath (Lin & McGarth, 1988) reported that CO relaxes vascular smooth muscle and dilates blood vessels by decreasing Ca^{2+} concentration in vascular smooth muscle. It has been suggested that CO induced cGMP-mediated relaxation by causing activation of K^+-channels and the resulting inhibition of voltage-operated Ca^{2+}-channels (Naseem et al, 2000). In a variety of different smooth muscles, hypoxia or metabolic inhibition has resulted in a significant reduction in force, but no change or increases in $[Ca^{2+}]_i$ either at rest or in activated tissues (Taggart & Wray, 1998). However, in our experiments with a high K^+-depolarized muscle, CO decreased muscle tension without changing $[Ca^{2+}]_i$ (Fig. 2). CO also did not inhibit both the resting tension (Fig. 1) and 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. However, further investigations are needed to draw any conclusion on the possible involvement of hypoxia or metabolic inhibition in CO-induced relaxation.

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 inhibits the Ca^{2+}-induced contraction by decreasing Ca^{2+} sensitivity of contractile elements (Nishimura & van Breemen, 1989; Pabelick et al, 2000). Like NO, CO is also known to increase intracellular cGMP levels (Utz & Ullrich, 1988), 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 downward (Fig. 3). Since CO shifted the $[Ca^{2+}]_i$-tension relationship downward, inhibition of high K^+-induced contraction by CO may be attributable to the decrease in the sensitivity of contractile elements to Ca^{2+}.

In smooth muscle, cGMP can regulate ion channels either by acting directly on channels or by acting indirectly as in activation of potassium channels (Abrahamsson, 1986). To address the nature of the cGMP-dependent relaxing effects of CO, we set out to explore the role of K^+ channels. CO has been shown to stimulate the outward K^+ current, hyperpolarizes the membrane potential, and elicits relaxation (Farrugia et al, 1998). However, in the present study, CO had little effect on the outward K^+ currents. In canine jejunum, heme oxygenase-2, an enzyme whose activity leads to the endogenous production of CO, was present in submucous plexus, in nerve fibers innervating the circular smooth muscle layer, and in epithelial cells in mucosa (Farrugia et al, 1998). In contrast, distribution of heme oxygenase-2 was only rarely observed in guinea pig ileal longitudinal smooth muscle cells (Maines, 1988). Therefore, the discrepancy between our results and the study by Farrugia et al (1998) may be partially due to the tissue difference, since the heme oxygenase distribution may be quite different between canine jejunal

circular muscle cells and guinea pig ileal longitudinal smooth muscle cells. It is therefore unlikely that K^+ channels activation is involved in the CO-induced relaxation in the guinra-pig ileum. Further study is needed to clarify this point.

In conclusion, the present study shows, for the first time, that CO inhibits smooth muscle contraction by decreasing Ca^{2+} sensitivity of contractile elements through cyclic GMP-dependent signaling pathways in guinea-pig ileal smooth muscle.

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