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The Effect of Carbon Monoxide on L-type Calcium Channel Currents in Human Intestinal Smooth Muscle Cells

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Carbon monoxide (CO) is low molecular weight oxide gas that is endogenously produced under physiological conditions and interacts with another gas, nitric oxide (NO), to act as a gastrointestinal messenger. The aim of this study was to determine the effects of exogenous CO on L-type calcium channel currents of human jejunal circular smooth muscle cells. Cells were voltage clamped with 10 mM barium (Ba^{2+}) as the charge carrier, and CO was directly applied into the bath to avoid perfusion induced effects on the recorded currents. 0.2% CO was increased barium current (I_{Ba}) by 15±2% (mean± S.E., $p<0.01$, n=11) in the cells. To determine if the effects of CO on barium current were mediated through the cGMP pathway, cells were pretreated with 1-*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, 10 μM), a soluble guanylyl cyclase inhibitor, and exogenous CO (0.2%) had no effect on barium currents in the presence of ODQ (2±1% increase, n=6, $p>0.05$). CO mediates inhibitory neurotransmission through the nitric oxide pathway. Therefore, to determine if the effects of CO on L-calcium channels were also mediated through NO, cells were incubated with N^G-nitro-L-arginine (L-NNA, 1 mM), a nitric oxide synthase inhibitor. After L-NNA pretreatment, 0.2 % CO did not increase barium current (4±2% increase, n=6, $p>0.05$). NO donor, SNAP (20 μM) increased barium current by 13±2% (n=6, $p<0.05$) in human jejunal smooth muscle cells. These data suggest that CO activates L-type calcium channels through NO/cGMP dependant mechanism.

Key Words: L-type calcium channels, Carbon monoxide (CO), Human intestinal smooth muscle cells

INTRODUCTION

Carbon monoxide (CO) can modulate many important physiological functions. Outside of gastrointestinal tract, CO modulates the release of hypothalamic hormones (Lamar et al, 1996; Mancuso et al, 1997), serves as a protective factor in hypoxia (Morita et al, 1997), and plays a role in long term potentiation in brain and in the regulation of cGMP level (Verma et al, 1993; Zhuo et al, 1993). Exogenous CO also modulates the vascular tension via inducing vasodilation in pulmonary artery (Villamor et al, 2000), renal resistance artery (Thorup et al, 1999) and aorta (Sammut et al, 1998) of rat. On the other hand, there was no intrinsic CO-mediated vasodilation in rat hepatic artery (Pannen & Bauer, 1998). CO increases the open probability of K^+ channel current and intracellular cGMP levels in isolated corneal epithelial cells (Rich et al, 1994), and increases the open probability of large conductance Ca^{2+} activated K^+ channels by acting on the histidine residue of α-subunit in rat tail artery smooth muscle cells (Wu et al, 2002).

In the gastrointestinal tract, CO relaxes the opossum intestinal anus sphincter (Rataan et al, 1993) and inhibits the contraction of guinea-pig ileum (Kwon et al, 2001).

CO increases potassium current in whole cell mode, hyperpolarizes the membrane potential, and increases cGMP level. The machinery for CO production (heme oxygenase-2) is present in canine jejunal smooth muscle cells, suggesting that CO may act as an inhibitory messenger in the gastrointestinal tract (Farrugia et al, 1998). CO and nitric oxide (NO) act as cotransmitters, and they are required for inhibitory neurotransmission in the mouse intestine (Xue et al, 2000) and activate soluble guanylate cyclase (sGC) and increase cGMP levels (Farrugia et al, 1998; Zyromski et al, 2001). NO has been reported to have opposing effects on L-type Ca^{2+} channels in intestinal smooth muscle cells of guinea pig (Kwon et al, 2000) and activates L-type Ca^{2+} channels of longitudinal smooth muscle cells in rat (Tanovic et al, 2001). However, the effect of CO for L-type Ca^{2+} channels has not yet been identified or characterized in human intestine. The aim of this study was to determine the effects of exogenous CO on L-type Ca^{2+} channel currents in human jejunal circular smooth muscle cells.

METHODS

Preparation of human jejunal circular smooth muscle cell

Human jejunal tissue was obtained from surgical tissue

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ABBREVIATIONS: CO, carbon monoxide; NO, nitric oxide; I_{Ba}, barium current; cGMP, cyclic guanosine 3',5'-monophosphate; NOS, nitric oxide synthase; sGC, soluble guanylyl cyclase; SNAP, S-nitroso-N-acetylpenicillamines.

during gastric bypass operations. Tissue specimens were harvested directly into chilled buffer, and dissociation was carried out with the use of a papain-based enzymatic technique (Farrugia et al, 1993). Briefly, the tissue was pinned down mucosal surface up in a Petri dish filled with normal Krebs solution. The mucosa was removed, and tissue was cut and placed serosal surface up in normal Krebs solution. Strips consisting of serosa and longitudinal muscle were removed and left the circular muscle layer and the submucosa. Strips of circular muscle were pulled off and cut into pieces. They were placed in enzyme solution containing 15 mg of papain (Sigma, USA) and 3 mg of dithiothreitol (Sigma, USA) in 15 ml of Hanks' solution (Sigma, USA) at 37°C and magnetically stirred.

After centrifugation, the tissue was mechanically dissociated to obtain single relaxed circular smooth muscle cells. The fresh isolated cells were used in electrophysiological recording within 6 hours of dissociation.

Patch clamp recordings and data analysis

Glass microelectrodes for whole-cell voltage clamp recordings were obtained by using borosilicate glass (World Precision Instruments, U.S.A) pulled on a Narishige electrode puller PP-83 (Narishige, Tokyo, Japan). Electrodes were coated with sylgard and fire polished to a final resistance of 3 to 5 MΩ. Currents were amplified, digitized, and processed using Axopatch 200B amplifier, Digidata 1322, and pCLAMP 8 software (Axon Instruments, Foster City, CA). Membrane currents were filtered at 2 kHz with an 8 pole Bessel filter, digitized and stored. No leakage subtraction was performed to the original recordings, and all data with visible changes in leakage currents during the course of study were excluded from further analysis. The cell was held at −90 mV and pulsed in 13 steps to voltages ranging from −90 mV to 30 mV. Each pulse was 500 milliseconds long. All electrophysiological experiments were carried out at room temperature (22~23°C). Data were expressed as means±standard error (S.E.). Differences before and after CO addition in the same cells were evaluated by Students *t*-test (two-tailed and paired), and the significant level of difference was determined (*$p<0.05$ or **$p<0.01$).

Solutions and chemicals

A bulb sealed with a rubber injection port was filled with CO at atmospheric pressure. A gas syringe was used to remove 1 ml of CO which was added to 100 ml of bath solution placed in another gas bulb. One hundred μL of 1% CO solution was gently added to the bath (500 μL) to 0.2% final concentration. CO solution was applied directly by syringe into bath solution to prevent mechanoactivation of L-type Ca^{2+} channel by continuous flow. CO solution was freshly prepared just before each experiment. N-omega-nitro-L-arginine (L-NNA), 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-

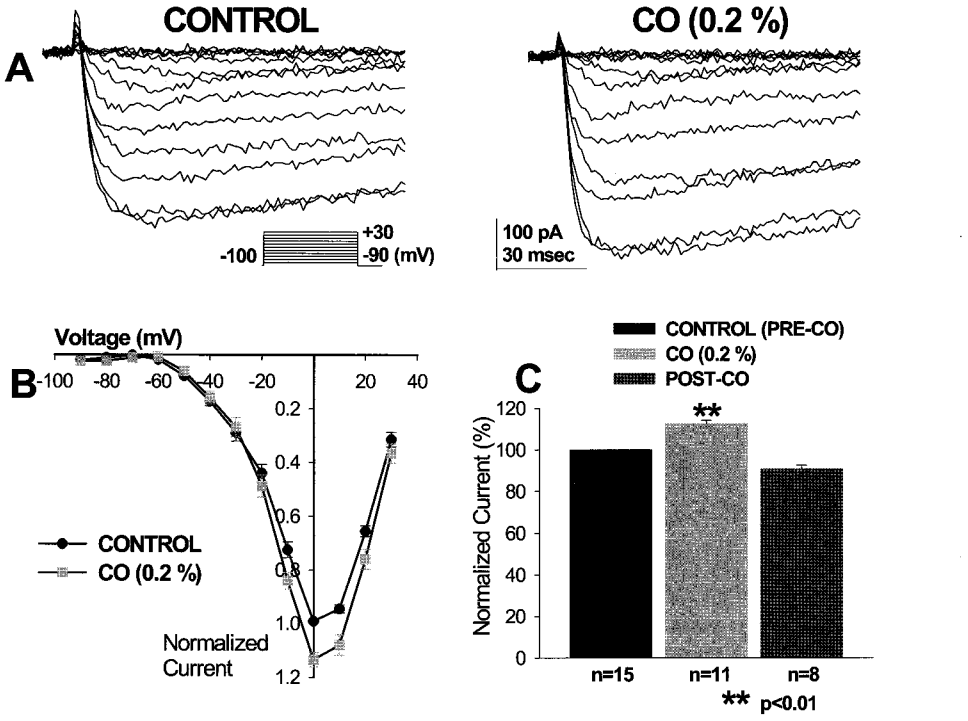

Fig. 1. The effect of exogenous carbon monoxide (CO) on the L-type Ca^{2+} currents of human jejunal circular smooth muscle cells. (A) Representative I_{Ba} in human jejunal smooth muscle cells is shown. CO (0.2%) induced a large increase in I_{Ba} (20.1%). (B) The normalized current-voltage (I-V) relationships of I_{Ba} before and after application of CO (n=11). (C) Bar graph shows the normalized mean maximal peak inward currents. Peak amplitude of I_{Ba} before CO addition was normalized as 100% (control). Data are means±S.E., **$p<0.01$ vs. control.

1-one (ODQ) and S-nitroso-N-acetylpenicillamines (SNAP) were purchased from Sigma (St. Louis, USA).

RESULTS

CO stimulates L-type Ca^{2+} channel current

To minimize outward potassium (K^+) current, the intrapipette solution contained 145 mM cesium (Cs^+) to block K^+ channels, and the bath solution contained 10 mM Ba^{2+} as the charge carrier since Ba^{2+} is more permeant to Ca^{2+} channels than Ca^{2+} itself.

The human jejunal circular smooth muscle cells were held at −90 mV, and the membrane potential was pulsed in 13 steps from −90 mV to 30 mV for 500 msec. The average cell capacitance (C_m) was 75.6±1.5 pF (n=38), and the access resistance (R_a) was 6.7±0.9 MΩ (n=35). The maximal inward current recorded in control cells was 226.0 ±13 pA (n=27), and the inward current (I_{Ba}) was blocked by nifedipine (1 μM), indicating L-type calcium channel current (Data not shown). Peak current of I_{Ba} typically increased during the first several minutes of recording before reaching a constant level. After reaching a constant level, CO at 0.2% final concentration, which is within the physiologic concentration in blood (Vreman et al, 1984), was added to the cells. Peak current of I_{Ba} was increased for a few minutes by the exogenous CO (0.2%) and then decreased. Significant increase of I_{Ba} to 15.1±1.7% (mean± S.E., n=11, $p<0.01$) by CO was reversed, when CO was washed out from the bath solution (Fig. 1), and no shift in the mean current−voltage relationships for the 11 cells was noted (Fig. 1).

In six other cells, amphotericin B-perforated patch clamp technique was applied to minimize the rundown of I_{Ba}. Under this condition, CO also increased the I_{Ba} by 11.5± 3.1% (n=6, $p<0.05$; data not shown), which was reversed after washout. There was no significant difference in the results obtained by either conventional whole-cell clamp or perforated patch clamp techniques. Next, the mechanism of the stimulatory effects of CO on L-type calcium channel in human jejunal smooth muscle cells was investigated.

Mechanism of CO stimulation on L-type Ca^{2+} channel current

The most important target of NO (Moncada et al, 1991) or CO (Morita et al, 1997) is soluble guanylate cyclase (sGC), to whose heme moiety they bind to activate the enzyme, thus increasing the intracellular concentration of cyclic guanosine 3', 5'-monophosphate (cyclic GMP). Cyclic GMP is a well-known mediator of the cellular effects produced by NO (Peng et al, 1996). Inhibitory mechanism of ODQ with a purified sGC has been suggested to be a slow, irreversible oxidation of the sGC haem iron to which NO binds (Schrammel et al, 1996).

As shown in Fig. 2, when the human jejunal myocytes were pretreated with ODQ at 10 μM concentration over 15 minutes, the stimulatory effect of CO on I_{Ba} was largely blocked, and there was no significant change in the I-V

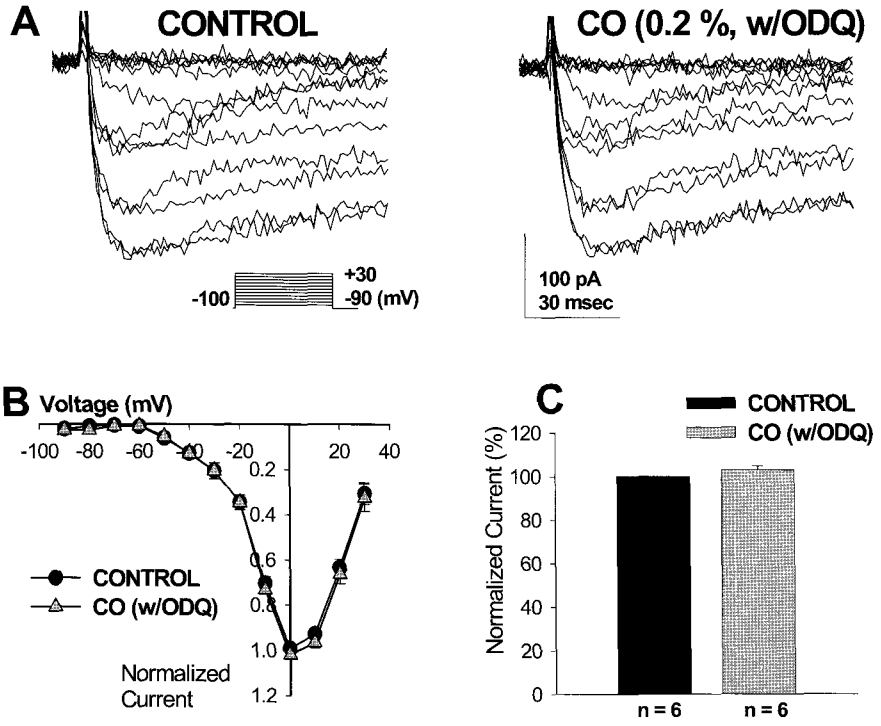

Fig. 2. Effect of CO after ODQ (10 μM) pretreatment. (A) Representative whole cell recording of human jejunal circular smooth muscle cells. The increase of I_{Ba} by the addition of CO (0.2%) was inhibited after pretreatment of bath solution with ODQ (10 μM). (B) Mean I-V relationships. There is no significant change in I-V relationship of the I_{Ba} (C) Normalized mean maximal peak inward current before and after CO addition with ODQ pretreatment.

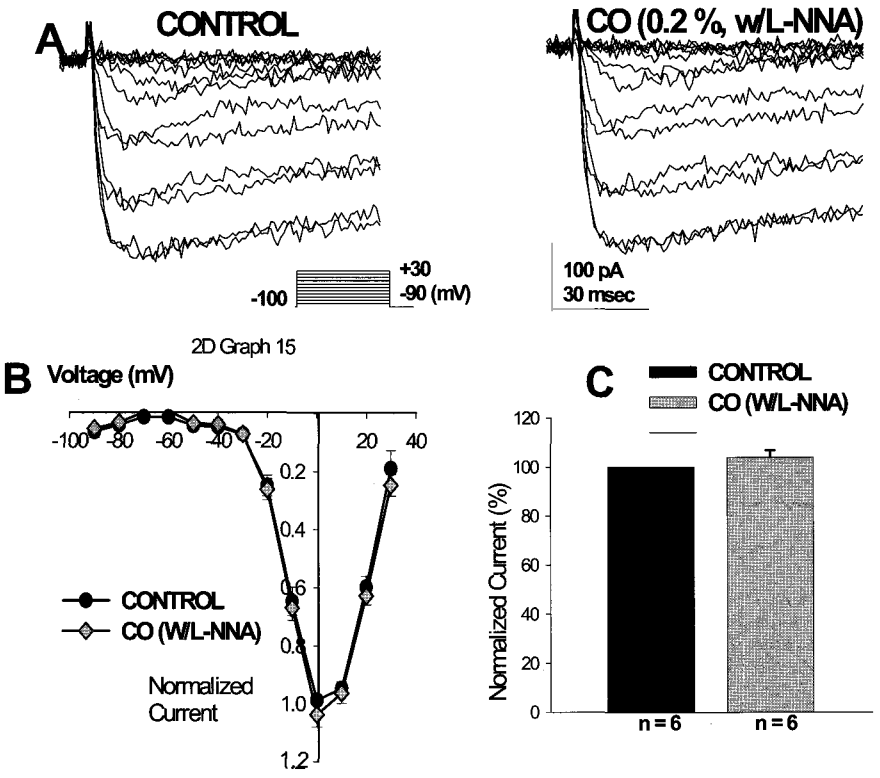

Fig. 3. Effect of CO after pretreatment with L-NNA (100 μM). (A) CO (0.2%) effect on I_{Ba} was reduced after 100 μM L-NNA pretreatment over 15 minutes. (B) Mean I-V relationship for the I_{Ba} is shown. After L-NNA pretreatment, exogenous CO did not make any significant change. (C) Normalized mean maximal peak inward currents before and after the addition of CO in L-NNA pretreatment state (4.1±2.8% in 6 cells, $p>0.05$ in human jejunal smooth muscle cells).

curve of the I_{Ba} between the control and CO added groups. These results suggested that CO increased L-type Ca^{2+} currents through cGMP pathway in human jejunal circular smooth muscle cells.

The effects of nitric oxide synthase (NOS) inhibitor, L-NNA (100 μM), was examined to verify whether NO pathway was involved in the action of CO on L-type Ca^{2+} currents: L-NNA is a classical competitive inhibitor with L-arginine and does not show significant isoform selectivity (Babu et al, 1998). As shown in Fig. 3, after pre-application of L-NNA over 15 minutes, the effect of CO on I_{Ba} was inhibited: I_{Ba} was increased by only 3.8±2.1% (n=6), and there was no significant change in the mean I-V curves in both groups ($p>0.05$).

The blocking effect of NOS inhibitors on the stimulatory effects of CO on I_{Ba} suggested that CO might be acting through the NOS/NO pathway. Therefore, whether NO donor could increase I_{Ba} was also tested. As seen in Fig. 4, SNAP (20 μM), a NO donor, increased I_{Ba} by 13.4±1.7% (n=6, $p<0.05$) and the positive effect of SNAP was prominent between −10 mV and +10 mV (n=6, $p<0.05$) in I_{Ba}. There was no significant shift in mean I-V curve by SNAP (Fig. 4).

DISCUSSION

This study reports that CO at a concentration of 0.2% increased the amplitude of inward current carried through L-type Ca^{2+} channel in human jejunal circular smooth muscle cells. It has been reported that exogenous CO at 1% concentration activates the K^+ current, induces hyperpolarization of membrane potential (Farrugia et al, 1993; 1998), and relaxes ileal smooth muscle of guinea pig through activation of guanylate cyclase (Utz & Ullrich, 1991). The main calcium entry pathway for human and canine jejunal circular smooth muscle cells is L-type Ca^{2+} channels, since nifedipine, an L-type Ca^{2+} channel blocker, blocks inward current (Farrugia et al, 1995). In the context of general inhibitory effects of CO, our initial hypothesis was that CO would directly inhibit L-type Ca^{2+} channel current (I_{Ba}) in human jejunal circular smooth muscle cell, however, the present results showed opposing effects, thus differing from a recent report that CO has no significant effect on I_{Ba} in ileal smooth of guinea pig (Kwon et al, 2001). However Kwon et al. (2001) used high concentrations (10%) of CO for the calcium current of guinea pig ileal muscle. It has been reported that CO may have dual effects, depending on the concentration: high levels of CO inhibit NOS activity and NO generation, but low concentration of CO induces NO release and may mimic the vascular effect of NO (Thorup et al, 1999). In the present study, I used low concentration of CO (0.2%), which is within physiological blood concentration (Vreman et al, 1984).

In the present study, without any pretreatments, CO variably increased I_{Ba} in most cells of human jejunal

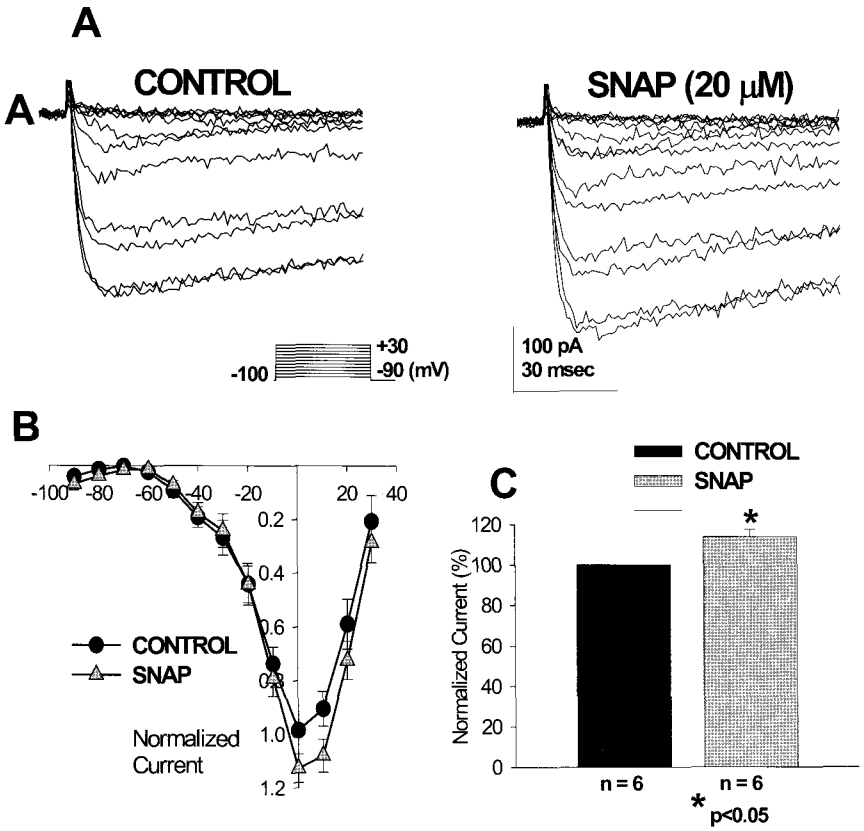

Fig. 4. SNAP increased the L-type Ca^{2+} channel current. (A) SNAP (20 μM) increased the I_{Ba} of human jejunal circular smooth muscle cells. (B) The normalized I-V relationships of the representative inward current before and after the application of SNAP. (C) Bar graph shows the summary of the results. It shows the significant increasing effect of SNAP. SNAP at 20 μM concentration increased I_{Ba} by 13.4±1.7% in 6 cells in human jejunal smooth muscle cells (n=6, *$p<0.05$).

circular smooth muscle, but decreased by −2.1 ~ −3.0% in 2 of the 11 cells, thus suggesting complicated and opposing mechanisms involved in the regulation of L-type calcium channel by CO. CO and NO are known for smooth muscle relaxant exerting their effect through sGC activation, but CO exhibits a poor vasorelaxant activity than NO (Villamor et al, 2000), and SNAP, a NO donor, is also reported to induce fast increase of cGMP concentration (Looms et al, 2002).

The blocking effects of ODQ (10 μM) indicate that cGMP is involved in the CO effect on I_{Ba}, which is in line with the previous reports that CO increased cGMP levels (Morita et al, 1995; Farrugia et al, 1998). The results also suggest that CO has little direct effect on I_{Ba} (Fig. 2).

CO may stimulate nitric oxide synthase (NOS) to release NO. The coexistence of NOS in gastrointestinal tracts with heme oxygenase that produce CO was immunohistochemically confirmed (Ny et al, 1997). Furthermore, CO induced NO release could be attributed to either stimulation of eNOS or NO displacement from a cellular storage pool (Thorup et al, 1999). In this study, when L-NNA, NOS inhibitor, was tested to elucidate whether CO directly activated sGC or acted through the NO synthesis pathway to produce cGMP, CO was found unable to increase I_{Ba} after L-NNA pretreatment, suggesting that CO acts mostly through NO pathway (Fig. 3). In concordance with this conclusion, SNAP, an NO releaser, also stimulated I_{Ba} in human jejunal circular smooth muscle cells (Fig. 4).

There are some discrepancies between CO induced weak vasorelaxation in the previous report and the increase of L-type calcium channel current in the present study. In the whole cell currents of smooth muscle cells in normal Ringer solution, K^+ currents are usually dominant and play crucial roles in determining smooth muscle tension. Therefore, the NO-mediated activation of Ca^{2+} -activated K^+ channels might be the major mechanism for the vascular relaxation in guinea pig aorta (Tanaka et al, 2000). Ca^{2+}-activated K^+ channel is one of the main causes of smooth muscle relaxation, and recent evidence indicates that changes in the channel activity are better described by alterations in subsarcolemmal Ca^{2+} levels than by overall or bulk cytosolic Ca^{2+} concentration (Stehnobittlel et al, 1992). Furthermore, the increase in the sarcolemmal Ca^{2+} concentration may be coupled to enhancement of Ca^{2+}-activated K^+ channel activity (Guia A et al, 1999). Therefore, activation of L-type Ca^{2+} channel current by CO might increase subsarcolemmal Ca^{2+} levels to activate Ca^{2+}-activated K^+ channel for vasorelaxation.

In conclusions, the present study suggests that 0.2% CO activates L-type Ca^{2+} channel in human jejunal circular smooth muscle cells through the generation of NO, and cGMP dependent mechanism.

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