Carbon monoxide activates human intestinal smooth muscle L-type Ca\(^{2+}\) channels through a nitric oxide-dependent mechanism

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\(^1\)Enteric Neuroscience Program, Mayo Clinic and Mayo Clinic College of Medicine, Rochester; \(^2\)Department of Physiology, College of Medicine, Chung-Ang University, Seoul, Korea; and \(^3\)Gastrointestinal Unit, Mayo Clinic and Mayo Clinic College of Medicine, Rochester, Minnesota

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Carbon monoxide (CO) activates human intestinal smooth muscle L-type Ca\(^{2+}\) channels through a nitric oxide-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 288:G7–G14, 2005. First published August 19, 2004; doi:10.1152/ajpgi.00205.2004.—Carbon monoxide (CO) is increasingly recognized as a physiological messenger. CO is produced in the gastrointestinal tract with diverse functions, including regulation of gastrointestinal motility, interacting with nitric oxide (NO) to mediate neurotransmission. The aim of this study was to determine the effect of CO on the human intestinal L-type Ca\(^{2+}\) channel expressed in HEK cells and in native cells using the patch-clamp technique. Extracellular solution contained 10 mM Ba\(^{2+}\) as the charge carrier. Maximal peak Ba\(^{2+}\) current (I\(_{\text{Ba}}\)) was significantly increased by bath application of 0.2% CO to transfected HEK cells (18 ± 3%). The NO donor S-nitroso-N-acetylpenicillamine also increased I\(_{\text{Ba}}\), and CO (0.2%) increased NO production in transfected HEK cells. The CO-induced increase in I\(_{\text{Ba}}\) was blocked when cells were pretreated with 1H-[1,2,4]-oxadiazolo[4,3-a]quinolxalin-1-one (10 \(\mu\)M) or inhibitors of NO synthase (NOS). The PKA inhibitor KT-5720 (0.5 \(\mu\)M) and milrinone (3 \(\mu\)M), a phosphodiesterase (PDE) III inhibitor, blocked the effect of CO on I\(_{\text{Ba}}\). Similar effects were seen in freshly dissociated human intestinal smooth muscle cells. The data suggest that exogenous CO can activate native and heterologously expressed intestinal L-type Ca\(^{2+}\) channels through a pathway that involves activation of NOS, increased NO, and cGMP but not through the mechanism of action involves the NO synthetic pathway.

Both CO and NO activate guanylyl cyclase resulting in the generation of cGMP (34). However, the affinity of CO to guanylyl cyclase is severalfold lower than for NO, suggesting that the effects of CO on guanylyl cyclase may require a sensitizing molecule (37) or that other pathways may be involved. Both CO and NO can directly modulate ion channels. NO directly modulates the \(\beta\)-subunit, and CO modulates the \(\alpha\)-subunit of large conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (42). NO also modulates L-type Ca\(^{2+}\) channel activity (4, 38, 44). The effects of NO on L-type Ca\(^{2+}\) channels are complex and tissue dependent. In the gastrointestinal tract, NO inhibits L-type Ca\(^{2+}\) channels from guinea pig Taenia coli (23) and canine gastric circular smooth muscle (31) but activates longitudinal smooth muscle L-type Ca\(^{2+}\) channels in rat intestine (39) and rat fundic longitudinal muscle accompanied by relaxation (14), likely reflecting the dual roles of Ca\(^{2+}\) as a second messenger and as an initiator of contraction. Recently, CO was shown to decrease prenatal rat ventricular myocyte outward current density and activate L-type Ca\(^{2+}\) channels (36) postnatally, suggesting that CO, like NO, may regulate L-type Ca\(^{2+}\) channel expression and/or function. The aim of the present study was to determine whether CO modulates intestinal smooth muscle L-type Ca\(^{2+}\) channels and whether the mechanism of action involves the NO synthetic pathway. We found that low levels of exogenous CO activated intestinal L-type Ca\(^{2+}\) channels through activation of NO synthase (NOS), and increased levels of NO and cGMP but not through PKG. Rather, the pathway appears to involve PDE III, partly by reducing cAMP breakdown through inhibition of phosphodiesterase (PDE) III.

MATERIALS AND METHODS

Human Jejunal Circular Smooth Muscle Cell Preparation

Human jejunal tissue, use of which was approved by the Institutional Review Board, was obtained as surgical waste tissue during gastric bypass operations performed for morbid obesity in otherwise

ion channels; gases; human studies; patch clamp

L-TYPE CA\(^{2+}\) CHANNELS PLAY A CENTRAL ROLE IN GASTROINTESTINAL SMOOTH MUSCLE CONTRACTILE ACTIVITY (18). BLOCK OF L-TYPE CA\(^{2+}\) CHANNELS REDUCES OR ABOLISHES INTESTINAL CONTRACTILE ACTIVITY. IT IS, THEREFORE, NOT SURPRISING THAT L-TYPE CA\(^{2+}\) CHANNEL ACTIVITY IS TIGHTLY REGULATED IN INTESTINAL SMOOTH MUSCLE. REGULATORY MECHANISMS INVOLVE CA\(^{2+}\), PH, CYCLIC NUCLEOTIDES, G PROTEINS AND A VARIETY OF EXTRACELLULAR LIGANDS (5).

Carbon monoxide (CO) is a low molecular weight gas that shares similar properties with another low molecular weight gas, nitric oxide (NO). CO, like NO, is generated under physiological conditions (26). The synthetic enzymes that produce CO, heme oxygenase 1 (HO1) and heme oxygenase 2 (HO2), are widely expressed in the gastrointestinal tract; thus CO is endogenously produced in the gastrointestinal tract (15, 27, 28). In the gastrointestinal tract, CO mediates nonadrenergic noncholinergic neurotransmission (43), sets the smooth muscle membrane potential gradient (43), and appears to protect against the development of postoperative ileus (29).

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healthy subjects. Tissue specimens were harvested directly into chilled buffer solution with warm ischemia times of ~30 s. Single, isolated circular smooth muscle cells were obtained from the human jejunal specimens as described previously (7). The freshly isolated cells were used for electrophysiological recording within 6 h of dissociation.

Transfection of HEK-293 Cells with L-type Calcium Channel Subunits

The human jejunal α1C(Cav1.2)- and β2-subunits were subcloned as an AgeI/Norf fragment from the cDNA library pSPORT1 (Invitrogen, Carlsbad, CA) cloning vector into pEGFP-C1 (Clontech, Palo Alto, CA) as previously published (24). Lipofectamine 2000 Reagent (Invitrogen) was used to transiently express the Ca^{2+} channel subunits in HEK-293 cells (American Type Culture Collection, Manassas, VA). Transfected cells were identified by their fluorescence microscopy and were patch clamped 48-56 h after transfection.

Patch-Clamp Recordings

Whole cell patch-clamp recordings were obtained by using Kimble KG-12 glass pulled on a P-87 puller (Sutter Instruments, Novato, CA). Electrodes were coated with R6101 (Dow Corning, Midland, MI) and fire polished to a final resistance of 3 to 5 MΩ. Currents were amplified, digitized, and processed by using a Axopatch 200A amplifier, a Digidata 1200, and pCLAMP 9 software (Axon Instruments, Foster City, CA). Data were filtered at 5 kHz with an eight-pole Bessel filter. The junction potential between pipette solution and bath solution was electronically adjusted to zero. Access resistance was recorded, and data was only used if access did not change by > 5 MΩ throughout the experiment. Records were obtained in both standard whole cell mode and amphotericin perforated, patch-clamp mode (see RESULTS). For standard whole cell records, rundown of the L-type Ca^{2+} current was evident within 10–15 min of recording. Rundown in amphotericin perforated, patch-clamp experiments was less marked. The time from application of CO to peak effect was typically <90 s. Whereas rundown may have masked the full effects of CO on the L-type Ca^{2+} current, the lack of a difference in the percent increase in current induced by CO between standard whole cell and amphotericin perforated patch-clamp experiments (see RESULTS) suggests that the effect of rundown on the observed results was not significant. Cells were held at -100 mV and pulsed for 512 ms in 13 steps to voltages ranging from -90 to +30 mV. Cells were returned to -100 mV between pulses with an interpulse interval of 1 s to allow complete recovery from inactivation. Current-voltage (I-V) curves were constructed by using the maximal peak inward current amplitude at each voltage. Data were normalized by using the formula $I_{\text{norm}} = 100(I_t)/(I_{\text{max}})$, where $I_{\text{norm}}$ is the normalized peak inward current at a particular voltage sweep, $I_t$ is the current at each voltage sweep, and $I_{\text{max}}$ is the maximum peak inward current from the set of traces (usually the peak inward current at 0 mV).

The pipette solution contained (in mM) 145 CsCl, 20 Cl-2, 2 EGTA, 5 HEPES, and 125 methanesulfonate (pH adjusted to 7.3 with CsOH). The bath solution contained (in mM) 10 BaCl2, 141.7 NaCl, 4.7 KCl, 166.4 Cl-2, 5 HEPES, and mannitol to reach a molarity of 290 mosM (pH adjusted to 7.35 with NaOH) for whole cell recordings. The 10 mM BaCl2 was used to increase the size of the current through L-type Ca^{2+} channels. All electrophysiological experiments were carried out at room temperature (22-23°C).

NO Measurement

NO production was measured by using the fluorescent NO indicator 4,5-diaminofluorescein (DAF-2) (2, 11, 16, 21). HEK-293 cells were passaged and plated onto 15-mm glass coverslips for transfection with the α1C- and β2-subunits of the L-type Ca^{2+} channel as described in Transfection of HEK-293 Cells with L-type Calcium Channel Subunits. At 48 h after transfection, the cells were washed twice and incubated for 10 min at 37°C with 10 μM DAF-2-DA (Sigma, St. Louis, MO) in OptiMEM (Invitrogen) supplemented with 0.1 mM L-arginine. The cells were washed again, resuspended in 1-ml OptiMEM, and viewed on an inverted epifluorescence microscope (model 51000TV; Zeiss, Germany) using light at 490 nm for excitation, and measuring the emitted light at 510 nm. Data were collected at 1-min intervals as 8-bit digital images using a SPOT camera (Diagnostic Instruments, Sterling Heights, MI). The fluorescence intensity was measured off-line for defined areas of interest in each image as grey-scale intensity with a custom macro written for the Zeiss KS400 software (Jim Tarara, Mayo Optical Morphology Core Facility, Rochester MN). Responses were quantified by measuring the initial slope of any observed change in fluorescence by linear regression (MS-Excel, Redmond, WA). The cells were preincubated with the NOS inhibitor N^ω-nitro-L-arginine (L-NAME, 1 mM) during DAF-2-DA loading to test the contribution of NOS to elevated NO levels. A positive control response was observed by adding bradykinin (1 μM) to the cells after loading (data not shown). CO was added from a 2% solution, to a final concentration of 0.2% as described in Chemicals and Data Analysis and the fluorescence was followed for 15 min. Specificity of DAF-2 fluorescence was established in preliminary experiments that demonstrated a linear relationship between DAF-2 fluorescence intensity in response to the calcium ionophore A23187 and with parallel control experiments using DAF-4, a nonfluorescent analog (data not shown). In addition, we tested the response of DAF-2 (100 μM in intracellular solution) to a saturated solution of CO (~2.3% vol/vol) and observed no change in fluorescence, whereas addition of NO reproducibly caused a large increase in emitted light. There was no apparent interaction between CO and NO, because the DAF-2 response to NO was not affected when CO was added before or after NO (data not shown).

Chemicals and Data Analysis

The CO solution was prepared fresh before each experiment as previously described (36). Briefly, a gas bulb with a rubber injection port was filled with CO (Scott Specialty Gases, Troy, MI) at atmospheric pressure. A glass gas syringe was used to remove 1 ml of CO, which was added to 100 ml of bath solution placed in another glass gas bulb. One hundred microliters of 1% CO solution was gently added to the bath (500 μl) to prevent mechanoactivation of L-type Ca^{2+} channels (6) for a final CO concentration of 0.2%, ~80 μM.

N^ω-nitro-L-arginine (l-NNA), KT-5823, and KT-5720 were purchased from Sigma-Aldrich; IH-[2,4]-oxadiazolo[4,3-g]-quinoxalin-1-one (ODQ), N-[3-(aminomethyl)benzyl] acetamidine·2HCl (1400W), and myristoylated PKC (20–28) were from Biomol (Plymouth Meeting, PA). 3-Bromo-7-nitroindazole (3-Br-7-NI), KT-5823, and KT-5720 were dissolved in DMSO; 1400W was dissolved in water. The final concentration of DMSO did not exceed 0.1%. Control experiments with DMSO at this concentration showed no effect on the l-type Ca^{2+} current. All drugs were added to the external 10 mM BaCl2 Ringer solution and were incubated with the cells for at least 15 min before data were recorded.

Data are expressed as means ± SE. Values from the same cells before and after addition of CO or the drug of interest were evaluated by Student’s t-test (two-tailed). A P value of <0.05 was considered significant.
RESULTS

HEK Cells

Effect of CO on L-type Ca$^{2+}$ channel current. The cell capacitance for HEK cells was 19 ± 0.9 pF (n = 80), and the access resistance was 9.6 ± 0.6 MΩ (n = 80). Peak Ba$^{2+}$ current ($I_{Ba}$) typically increased during the first several minutes of recording before reaching steady state. After a minute at steady state, CO, at a final concentration of 0.2%, was added to the bath. Exogenous CO resulted in an increase in $I_{Ba}$ recorded from expressed human intestinal Ca$^{2+}$ channels in transfected HEK-293 cells (Fig. 1). CO (0.2%) increased $I_{Ba}$ by 18 ± 3% (n = 21, P < 0.01); 19 of 21 cells showed an increase in $I_{Ba}$; in the other two cells, $I_{Ba}$ was unchanged. There was no shift in the voltage dependence of the I-V relationship (Fig. 1). The increase in current evoked by CO was reversible on washout of CO (Fig. 1). The maximal solubility of CO in water is 2.3:100 ml, or by our nomenclature, 2.3%; 100 μl was added to a 500-μl bath resulting in a sixfold dilution. Therefore, the maximal concentration of CO that can be applied in this configuration is 0.38%. CO (0.38%) increased $I_{Ba}$ by 21 ± 6% (n = 5, P < 0.005).

To determine whether the observed effects of CO were secondary to differential washout of regulators of the L-type Ca$^{2+}$ current, we used the amphotericin B-perforated patch-clamp technique. In these experiments, CO (0.2%) also increased $I_{Ba}$ (24 ± 9%, n = 6, P < 0.05).

Effect of inhibition of cGMP formation. CO activates guanylyl cyclase, resulting in an increase in cGMP levels (25, 41). To determine whether the observed effects of CO on the L-type Ca$^{2+}$ current were through a cGMP pathway, we used ODQ to inhibit soluble guanylyl cyclase (12). Transfected HEK cells were pretreated with ODQ (10 μM) for 15 min, patch clamped, and after steady-state $I_{Ba}$ was reached, CO (0.2%) was added to the bath. In the presence of ODQ, CO had no effect on $I_{Ba}$ (CO changed $I_{Ba}$ by 4 ± 3%; n = 14, P > 0.05, Fig. 2).

Effect of NOS inhibitors and effect of S-nitroso-N-acetylpenicillamine, an NO donor. The above results suggested that the mechanism of action of CO on L-type Ca$^{2+}$ channels was through the guanylyl cyclase/cGMP pathway. NO, like CO, is a diatomic gas whose predominant mechanism of action is also through the guanylyl cyclase/cGMP pathway. We used relatively selective inhibitors of the isoforms of NOS (1) to determine whether the observed effects of CO on $I_{Ba}$ involved NO. Cells were incubated with the inhibitor for 15 min before CO was added. 1400W was used as an inhibitor of inducible NOS (iNOS) (13). Inhibition of iNOS did not significantly inhibit the stimulatory effects of CO on $I_{Ba}$. CO (0.2%) added to the bath after pretreatment with 1400W (100 nM) increased $I_{Ba}$ by 18 ± 7% (n = 10, P < 0.05) in transfected HEK-293 cells. Inhibition of neuronal NOS (nNOS) by 3 Br 7-NI (1 μM, 15 min), a relatively selective nNOS inhibitor (30) also did not significantly inhibit the stimulatory effects of CO on $I_{Ba}$ (16 ± 6%, n = 11, P < 0.5). There are no relatively selective endothelial NOS (eNOS) inhibitors. L-NIO has been previously used as a relatively selective eNOS inhibitor although current data suggest that L-NIO also blocks iNOS (1). The effect of CO on $I_{Ba}$ was significantly reduced by L-NIO (5 μM, 15 min, 11 ± 5%, n = 10, P > 0.05, data not shown). The lack of effect of the relatively selective iNOS and nNOS inhibitors suggests that the predominant effect of CO may be through eNOS.

Reduction in the stimulatory effects of CO on $I_{Ba}$ NOS inhibition suggested that CO may be acting through the NOS/NO pathway. If that is the case, then NO donors should also increase $I_{Ba}$. Addition of the NO donor S-nitroso-N-acetylpenicillamine (SNAP; 20 μM) increased $I_{Ba}$ by 24 ± 4% (n = 6, P < 0.05, Fig. 3).

Stimulation of NO release by CO. Addition of CO (0.2%) caused a prominent increase in DAF-2 fluorescence consistent with production of NO in transfected HEK-293 cells (Fig. 4). The effect was significantly inhibited by preincubation of the cells with the nonspecific NOS inhibitor l-NAME (1 mM) as determined by measurement of the slope of the increase in fluorescence after CO addition (P < 0.05, n = 23 cells).

Effect of protein kinase inhibitors. cGMP activates PKG, which, in turn, results in phosphorylation of effectors, including ion channels. To determine whether the stimulation of $I_{Ba}$ by CO in transfected HEK-293 cells was mediated by activation of PKG, we studied the effect of KT-5823, a specific PKG inhibitor, on expressed $I_{Ba}$. In the presence of KT-5823 (1 μM, 15 min), CO increased $I_{Ba}$ by 14 ± 5% (n = 7, P < 0.05), suggesting that the PKG pathway was not a major mediator of the stimulatory effect of CO on $I_{Ba}$. cGMP may also activate cAMP-dependent PKA through cross talk (20). KT-5720, a selective inhibitor of PKA, was used to determine whether the increase in cGMP generated in the presence of CO activated PKA. CO had no effect on $I_{Ba}$ in the presence of KT-5720 (0.5

Fig. 1. Effect of exogenous carbon monoxide (CO) on the L-type Ca$^{2+}$ channel currents in transfected HEK-293 cells. A: inward currents were recorded from HEK-293 cells transfected with human intestinal smooth muscle α1C(Cav1.2)- and β2-subunits. CO (0.2%) increased maximal peak Ba$^{2+}$ current ($I_{Ba}$) by 18 ± 3% (n = 21, P < 0.001). B: normalized current-voltage relationships are shown [control transfected HEK-293 cells (•), CO (○)]. C: normalized $I_{Ba}$ before and during CO exposure and after removal of CO from the bath (*P < 0.05).
Fig. 2. Block of the effect of CO after pretreatment with 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). A: in the presence of ODQ (10 μM, 15 min), exposure to CO (0.2%) had no effect on \( I_{Ba} \) in HEK-293 cells transfected with the human jejunal \( \alpha_\text{I(C)} \)-and \( \beta_2 \)-subunits. B: mean current voltage relationships [transfected HEK-293 cells (\( \bullet \)), CO (\( \circ \)) and the normalized \( I_{Ba} \).

Fig. 3. Increase in L-type Ca\(^{2+} \) channel current induced by the nitric oxide (NO) donor \( S \)-nitroso-\( N \)-acetylpenicillamine (SNAP). A: NO donor SNAP increased \( I_{Ba} \) in transfected HEK-293 cells (10 μM). B: mean current voltage relationships [transfected HEK-293 cells (\( \bullet \)), with SNAP (\( \circ \))]. C: normalized \( I_{Ba} \). (*\( P < 0.05 \)).
Human Jejunal Circular Smooth Muscle Cells

We also tested the effects of CO on the native L-type Ca\(^{2+}\) current in freshly dissociated human jejunal circular smooth muscle cells. Human jejunal circular smooth muscle cells had a capacitance of 71 ± 2 pF (n = 69). The access resistance was 7 ± 0.4 MΩ (n = 69). Exogenous CO resulted in an increase in \(I_{Ba}\) in human jejunal circular smooth muscle cells by 14 ± 2% (n = 21, P < 0.01, Fig. 5) in standard whole cell recordings and by 12 ± 3% (n = 7, P < 0.05) in amphotericin B-perforated patch-clamp recordings. As with transfected HEK cells, no shift in the mean current-voltage relationships was noted, and the effects of CO were reversible on washing out CO from the bath solution. The soluble guanylyl cyclase inhibitor ODQ blocked the effects of 0.2% CO (3 ± 2% increase, n = 9, P > 0.05, Fig. 5). Similar to transfected HEK cells, 1400W (100 nM) did not completely block the stimulatory effects of CO (7 ± 1%, n = 6, P > 0.05), but in contrast to transfected HEK cells, 3 Br 7-NI (1 μM, 15 min) reduced the CO effect on \(I_{Ba}\) in human jejunal circular smooth muscle cells (5 ± 2%, n = 6, P > 0.5). L-NIO (5 μM, 15 min) blocked the CO effect of \(I_{Ba}\) in human jejunal circular smooth muscle cells (3 ± 1%, n = 6, P > 0.05), suggesting involvement of nNOS and possibly eNOS in the stimulatory effects of CO in human jejunal circular smooth muscle cells. The NO donor SNAP increased \(I_{Ba}\) by 14 ± 4% (n = 6 P < 0.05). We also tested the effects of protein kinase inhibitors on the stimulatory effect of CO on \(I_{Ba}\). In the presence of the PKG inhibitor KT-5720 (1 μM, 15 min), CO increased \(I_{Ba}\) in human jejunal circular smooth muscle cells by a small but significant amount (5 ± 1%, n = 9, P < 0.05). KT-5720 (0.5 μM, 15 min), a PKA inhibitor, blocked the effects of CO on \(I_{Ba}\) (2 ± 2% increase, n = 7, P > 0.05). Milrinone (3 μM), the PDE III inhibitor, blocked the increase in \(I_{Ba}\) evoked by 0.2% CO (1 ± 1%, n = 10, P > 0.05).

DISCUSSION

The main finding of this study is that exogenous CO stimulated L-type Ca\(^{2+}\) channels in human intestinal smooth muscle cells and expressed in HEK-293 cells through a pathway that involved stimulation of NOS, generation of NO, activation of guanylyl cyclase, an increase in cAMP, and activation of PKA. Whereas CO stimulated both native L-type Ca\(^{2+}\) channels in human jejunal circular smooth muscle cells and expressed channels in HEK cells, some differences were noted. The NOS inhibitor 3 Br 7-NI did not prevent the increase in \(I_{Ba}\) in transfected HEK cells but did in native cells most likely reflecting differences in intracellular molecules between the two cell types. CO has a multitude of functions in the gastrointestinal tract including participating in inhibitory neurotransmission (43), setting the smooth muscle membrane potential gradient (8), activating K\(^+\) channels, and modulating inflammation (15, 26, 29). The present study suggests that CO can also increase current through L-type Ca\(^{2+}\) channels, reflecting the diverse effects of CO in the gastrointestinal tract. Activation of L-type Ca\(^{2+}\) channels by CO has been recently reported in ventricular myocytes. Prenatal exposure of pregnant rats to 150 ppm CO results in an increase in L-type Ca\(^{2+}\) channel current density in 4-wk-old pups born from the exposed rats but not at 2 or 8 wk (36). NO also modulates intestinal smooth muscle L-type Ca\(^{2+}\) channels with both activation (39) and inhibition (23), although measurement of direct activation of Ca\(^{2+}\) channel currents was not carried out. The physiological effects of the action of CO on intestinal L-type Ca\(^{2+}\) channels is difficult to predict. A focal increase in intracellular Ca\(^{2+}\) may activate Ca\(^{2+}\)-activated conductances such as large-conductance Ca\(^{2+}\)-activated K channels, which may result in relaxation, whereas a more global increase may activate the contractile apparatus.

The relationship between CO and NO is complex (15). CO shares similar molecular weight and solubility characteristics with NO (9). As an endogenously produced gas, CO can activate guanylyl cyclase to produce cGMP, but its potency is considerably less than NO (41). The apparent disparity between the observed ability of CO to increase cGMP levels and the in vitro studies that suggest that CO is at best a weak direct activator of guanylyl cyclase has led to the suggestion that endogenous guanylyl cyclase may be sensitized to CO (10, 37). Cross talk between the NO and CO signaling pathways occurs
at multiple levels. NO increases HO1 expression by inducing transcription and stabilizing HO mRNA (17) as well as through a cGMP-dependent pathway (32). CO regulates NOS activity in a concentration-dependent manner with high CO levels inhibiting NOS activity and low CO levels increasing NO production (40). CO is reported to induce directly release of NO from a cellular storage pool (40). Our data show that NOS inhibitors blocked the CO-induced activation of L-type Ca2⁺/H11001, suggesting that CO may not only induce release of preformed NO but also stimulates NO synthesis. The finding that CO can stimulate NO production provides another mechanism of action for CO and perhaps may explain the observed disparity between measured levels of cGMP induced by CO, and the known low affinity of guanylyl cyclase for CO as CO may indirectly increase cGMP levels through NO. The present studies suggest that CO can, in fact, activate the nNOS/NO/cGMP pathway, confirming that at low concentrations, CO is as an agonist of NO production.

The predominant mechanism of action of the effects of CO on L-type Ca2⁺ channels appeared to be through cAMP not cGMP. The direct effect of cGMP through PKG on L-type Ca2⁺ channels has been well studied and reviewed (20). The predominant effect of PKG activation by cGMP is inhibition of L-type Ca2⁺ channels (3, 19) either by direct or indirect phosphorylation of the α-subunit or through increased production of PDE II, resulting in increased breakdown of cAMP and, therefore, reduced PKA levels. It is possible that CO had a dual effect on L-type Ca2⁺ channels in human intestinal smooth muscle, inhibiting channel activity through cGMP and increasing channel activity through cAMP. However, inhibition of PKG did not further increase the stimulatory effects of CO on native and expressed human intestinal L-type Ca2⁺ channels over the stimulation seen in the absence of PKG inhibitors, suggesting that the cGMP/PKG pathway was not a major regulatory pathway for the effects of CO on L-type Ca2⁺ channels in either of these cell types. In contrast, inhibition of PKA did block the effects of CO on the L-type Ca2⁺ channels implicating this pathway in CO stimulation of L-type Ca2⁺ channels. Cross talk exists between the PKG and PKA pathways (reviewed in Ref. 20). Elevated cGMP levels can activate directly PKA as both cGMP and cAMP are not absolutely specific for their respective kinases. cGMP also inhibits PDE III. Inhibition of PDE III results in decreased breakdown of cAMP (33), resulting in an elevation of endogenous cAMP levels and further stimulation of PKA. The effects of PKA on intestinal smooth muscle L-type Ca2⁺ channels has been reported predominantly, but not exclusively, to be stimulatory, similar to its effects in cardiac myocytes. However, PKA stimulation of intestinal L-type Ca2⁺ channels is reported to be less pronounced than PKA stimulation of cardiac myocytes and does not left-shift the I-V relationship (reviewed in Ref. 20). Our findings are also in agreement with these observations,
with a modest increase in $I_{\text{Ba}}$ and no shift in the $I-V$ relationships.

Whether endogenously produced CO regulates L-type Ca$^{2+}$ channels in intestinal smooth muscle is not known. Our experiments utilized low concentrations of exogenous CO. The major source of endogenous CO is through the heme breakdown pathway catalyzed by heme oxygenase. Under unstimulated conditions, HO2 is the predominant isofrom expressed in the gastrointestinal tract (26). HO1 is expressed ubiquitously in mammalian cells and is upregulated in response to a large variety of stimuli, including inflammation (26). HO2 is expressed predominantly in enteric nerves, interstitial cells of Cajal, and mucosal epithelial cells of mouse (27). Endogenously produced CO can modulate neurotransmission and act as a hyperpolarizing factor (43), suggesting that enough CO is produced to have a physiological effect. However, the microenvironment around the L-type Ca$^{2+}$ channel will determine whether CO also functions as a regulator of L-type Ca$^{2+}$ channels in vivo as well as a modulator of neurotransmission, inflammation, Ca$^{2+}$ sparks, and as a hyperpolarizing factor.

In summary, our data suggest that low levels of exogenous CO can activate native and heterologously expressed intestinal L-type Ca$^{2+}$ channels through a pathway that involves activation of NOS, increased NO levels, and increased cGMP but does not appear to involve PKG. Rather, the wayward pathways appear to involve PKA, partly by reducing cAMP breakdown through inhibition of PDE III. CO-induced NO production may explain the apparent discrepancy between the low affinity of guanylyl cyclase for CO and the robust cGMP production evoked by CO.

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