Effects of carbon monoxide on ion transport across rat distal colon

Julia Steidle and Martin Diener

Institute for Veterinary Physiology, Justus-Liebig University, Giessen, Germany

Submitted 10 September 2010; accepted in final form 14 November 2010

Steidle J, Diener M. Effects of carbon monoxide on ion transport across rat distal colon. Am J Physiol Gastrointest Liver Physiol 300: G207–G216, 2011. First published November 18, 2010; doi:10.1152/ajpgi.00407.2010.—The aim of the present study was to investigate whether carbon monoxide (CO) induces changes in ion transport across the distal colon of rats and to study the mechanisms involved. In Ussing chamber experiments, tricarbonyl dichlororuthenium(II) dimer (CORM-2), a CO donor, evoked a concentration-dependent increase in short-circuit current (Isc). A maximal response was achieved at a concentration of 2.5·10^{-4} mol/l. Repeated application of CORM-2 resulted in a pronounced desensitization of the tissue. Anion substitution experiments suggest that a secretion of Cl^{-} and HCO_{3}^{-} underlie the CORM-2-induced current. Glibenclamide, a blocker of the apical cystic fibrosis transmembrane regulator channel, inhibited the Isc induced by the CO donor. Similarly, bumetanide, a blocker of the basolateral Na^{+}-K^{+}-2Cl^{-} cotransporter, combined with 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid sodium salt, an inhibitor of the basolateral Cl^{-}/HCO_{3}^{-} exchanger, inhibited the CORM-2-induced Isc. Membrane permeabilization experiments indicated an activation of basolateral K^{+} and apical Cl^{-} channels by CORM-2. A partial inhibition by the neurotoxin, tetrodotoxin, suggests the involvement of secretomotor neurons in this response. In imaging experiments at fura-2-loaded colonic crypts, CORM-2 induced an increase of the cytosolic Ca^{2+} concentration. This increase depended on the influx of extracellular Ca^{2+}, but not on the release of Ca^{2+} from intracellular stores. Both enzymes for CO production, heme oxygenase I and II, are expressed in the colon as observed immunohistochemically and by RT-PCR. Consequently, endogenous CO might be a physiological modulator of colonic ion transport.

Ca^{2+}, carbon monoxide; Cl^{-} secretion; heme oxygenase I and II

THE INTESTINAL EPITHELIUM is able to absorb and to secrete water and electrolytes (2). Different pathways are involved in the regulation of the switch between both transport directions. Classical neurotransmitters and hydrophilic hormones bind to membrane-bound receptors and thereby activate different signaling transduction pathways within the epithelial cells. Recently, it has been shown that small gaseous molecules also regulate epithelial functions. Nitric oxide (NO) was the first gasotransmitter to be described (49). This molecule is produced from the amino acid arginine in a reaction that is catalyzed by three isoforms of NO synthase. NO exerts its actions mainly via activation of the soluble guanylate cyclase, producing the second-messenger guanosine 3',5'-cyclic monophosphate (cGMP) (23). Increased production of NO in the colon results in a strong Cl^{-} secretion, caused by the opening of apical Cl^{-} and basolateral K^{+} channels, as well as stimulation of the Na^{+}-K^{+} pump (39).

In contrast, another gaseous molecule, namely carbon monoxide (CO) is often considered only as a respiratory toxin. However, CO is a gasotransmitter apparently also involved in the regulation of several cell functions. For example, CO has pronounced actions on blood circulation, as it induces vasodilatation (22). CO is produced during the degradation of hemoglobin. This process is catalyzed by two enzymes: the inducible isoform heme oxygenase I (HO-I) and the constitutive isoform HO-II (26). A third isoform, HO-III, has been cloned from rat tissue (27), but its functional significance has been doubted (19), because HO-III catalyzes heme degradation to a much smaller degree than the other two enzymes. CO, as a small gaseous molecule, is able to cross membranes via diffusion. Obviously, CO, like NO, can stimulate the soluble guanylate cyclase and thereby stimulate the intracellular production of cGMP (14). The effects of this second messenger are mediated either by an activation of protein kinase G (7), or by an inhibition of cGMP-sensitive phosphodiesterases (32). Furthermore, CO is able to activate K^{+} channels directly leading to a hyperpolarization of the membrane (22). In H441 cells, a human bronchiolar epithelial cell line, CO inhibits Na^{+} channels independently from cGMP. This effect may be caused by a modification of histidine residues in the ion channels or by regulators of the ion channels, as concluded from experiments with diethyl pyrocarbonate, a histidine-modifying agent (1).

Enteric neurons, the key players in the regulation of intestinal transport, express the enzymes for CO production. For example, HO-II is found in the human myenteric plexus (28), as well as in the myenteric and submucosal plexus of the rat ileum (9). Thus CO apparently acts as a gasotransmitter in the enteric nervous system, which is able to regulate the gastrointestinal functions largely independently from the central nervous system (52). In Caco-2 cells, i.e., a colonic tumor cell line, a CO donor or pretreatment with heme to stimulate endogenous CO production causes Cl^{-} secretion (47). There is, however, no information available about the effects of CO on native intestinal epithelium. Therefore, the aim of the present study was to investigate whether CO induces changes in ion transport across rat colonic mucosa and to study the mechanisms involved.

MATERIAL AND METHODS

Animals. For Ussing chamber and immunohistochemical experiments, female and male Wistar rats with a body mass of 200–250 g were used. Imaging experiments were conducted with Wistar rats with a body mass of 110–140 g. The animals were bred and housed at the institute for Veterinary Physiology of the Justus-Liebig University at an ambient temperature of 22.5°C and air humidity of 50–55% on a 12:12-h light-dark cycle with free access to water and food until the time of the experiment. Experiments were approved by Regierungspräsidium Giessen, Giessen, Germany.

Solutions. The experiments with isolated crypts were conducted with the following buffers. The EDTA (ethylenediaminetetraacetic acid) solution for the crypt isolation contained the

0193-1857/11 Copyright © 2011 the American Physiological Society
following (in mmol/l): 107 NaCl, 4.5 KCl, 25 NaHCO3, 1.8 Na2HPO4, 0.2 Na2HPO4, 0.1 EDTA, 12.2 glucose with 1 g/l bovine serum albumin (BSA). The solution was gassed with carbogen and kept at a temperature of 37°C. The pH of the solution was adjusted to 7.4 by Tris base (tris(hydroxymethyl)-aminomethane). The high K+ Tyrode for the storage of the crypts consisted of the following (in mmol/l): 100 potassium gluconate, 30 KCl, 20 NaCl, 1.25 CaCl2, 1 MgCl2, 10 HEPES, 12.2 glucose, 5 sodium pyruvate, and 1 g/l BSA. The solution was adjusted with KOH to a pH of 7.4. The fura-2 experiments were performed using a Tyrode solution of the following composition (in mmol/l): 140 NaCl, 5.4 KCl, 1.25 CaCl2, 1 MgCl2, 10 HEPES, and 12.2 glucose, pH of 7.4, adjusted by NaOH/HCl. For the Ca2+-free buffer, CaCl2 was omitted in this buffer without administration of a chelator.

Most of the Ussing chamber experiments were carried out in a bathing solution containing the following (in mmol/l): 107 NaCl, 4.5 KCl, 25 NaHCO3, 1.8 Na2HPO4, 0.2 Na2HPO4, 1.25 CaCl2, 1 MgSO4, and 12.2 glucose. The solution was gassed with 5% (vol/vol) CO2 and 95% (vol/vol) O2, temperature at 37°C, and had a pH of 7.4 (adjusted by NaHCO3/HCl). For the Cl−-free buffer, NaCl and KCl were replaced equimolarly by sodium gluconate and potassium gluconate. For the HCO3−-free buffer, a HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid)-buffered Tyrode solution (see above) was used. When, in addition Cl− was omitted, NaCl was substituted by sodium gluconate. All HEPES-buffered solutions were gassed with O2 in the Ussing chamber experiments. For the depolarization of the basolateral membrane, a 111.5 mmol/l KCl solution was used, in which NaCl of the HCO3−-buffered solution was replaced by equimolar KC1 on the serosal side. On the mucosal side, the solution was a 107 mmol/l sodium gluconate/4.5 mmol/l potassium gluconate buffer. During the experiments with nystatin, a buffer was used at the mucosal side in which the 140 mmol/l NaCl/4.5 mmol/l KC1 of the standard HEPES-buffered Tyrode were replaced by 131 mmol/l NMDG (N-methyl-d-glucamine) Cl and 13.5 mmol/l KCl Tyrode solution.

For the immunohistochemical experiments, a 100 mmol/l phosphate buffer was used containing the following (in mmol/l): 80 Na2HPO4 and 20 NaH2PO4; pH was 7.4 (adjusted by NaOH/HCl).

Ussing chamber experiments. The serosa and muscle layers were stripped away by hand to obtain a mucosa-submucosa preparation from the distal colon. Briefly, the colon was placed on a small plastic rod with a diameter of 5 mm. A circular incision was made near the anal end with a blunt scalpel, and the serosa, together with the muscle layers, were gently removed in a proximal direction.

The mucosa-submucosa preparation was fixed in a modified Ussing chamber bathed with a volume of 3.5 ml on each side of the mucosa. The tissue was incubated at 37°C and short-circuited by a computer-controlled, voltage-clamp device (Ingenuer Buehfor Mess und Datentechnik MUESSLER, Aachen, Germany) with correction for solution resistance. Tissue conductance was measured every minute by the voltage deviation induced by a current pulse (250 μA, duration 200 ms) under open-circuit conditions. Short-circuit current (Isc) was continuously recorded on a chart recorder. Isc is expressed as microequivalents per hour per centimeter squared, i.e., the flux of a monovalent ion per time and area, with 1 μeq h⁻¹·cm⁻² = 26.9 μA/cm². In Table 2, the maximal increase in Isc induced by the CO donor, tricarboxylicdichlororuthenium(II) dimer (CORM-2), is given as the difference to the baseline just before administration of the CO donor.

For the initial concentration-response experiments, CORM-2 was administered in increasing concentrations to an individual tissue. The compartment where the CO donor had been applied was exchanged three times with five times the chamber volume, before the next concentration of the CO donor was administered. In all further experiments, CORM-2 was administered only once to an individual tissue to avoid desensitization. In general, one tissue served to measure the control response evoked by CORM-2, and the other was treated with putative antagonists before CORM-2 was applied. If the antagonist had to be administered in a solvent, the control tissue was also pretreated with the solvent.

Measurement of apical and basolateral ion currents. The apical membrane was permeabilized by mucosal administration of the ionophore, nystatin (100 μg/ml) dissolved in dimethyl sulfoxide (DMSO; final concentration 0.2%, vol/vol). Nystatin was ultrasonified immediately before use. The Isc response to the ionophore was tested in the presence of a K+ gradient, but in the absence of mucosal Na+ to avoid a contribution of the basolateral Na+−K+ pump to the measured Isc (140 mmol/l NaCl/4.5 mmol/l KCl HEPES-buffered Tyrode at the serosal side, 131 mmol/l NMDG Cl/13.5 mmol/l KCl Tyrode at the mucosal side). To depolarize the basolateral membrane, the tissue was exposed to a high K+ buffer (111.5 mmol/l KC1) at the serosal side. Under these conditions, the basolateral membrane is electrically eliminated (11, 34), and changes in Isc reflect changes in current across the apical membrane.

Imaging experiments. For the fura-2 experiments, intact colonic crypts were isolated. The mucosa-submucosa preparation was fixed on a plastic holder with tissue adhesive and transferrered for ~7 min to the EDTA solution. The mucosa was vibrated once for 30 s to obtain crypts (37). They were collected in a high K+ glucylate Tyrode buffer.

Relative changes in the intracellular Ca2+ concentration were measured using fura-2 (Molecular Probes, Leiden, The Netherlands), a Ca2+−sensitive fluorescent dye (17). The crypts were pipetted into the experimental chamber with a volume of ~3 ml. The crypts were attached to the glass bottom of the chamber with the aid of poly-l-lysine (0.1 g/l; Biochrom, Berlin, Germany). They were incubated for 60 min with 3 μmol/l fura-2 acetoxymethyl ester. The dye ester not taken up by the cells was then washed away. The preparation was superfused hydrostatically throughout the experiment with 140 mmol/l NaCl Tyrode. Perfusion rate was ~250 ml/h.

Changes in the cytosolic Ca2+ concentration were registered as changes in the fura-2 ratio (emission at an excitation wave length of 340 nm divided by the emission at an excitation wave length of 380 nm). Experiments were carried out on an inverted microscope (Olympus IX-50) equipped with an epifluorescence setup and an image analysis system (Till Photonics, Martinsried, Germany). The emission >470 nm was measured from the regions of interest. Data were sampled at 0.2 Hz. The baseline in the fluorescence ratio of the fura-2 signal was measured for several minutes before drugs were administered.

Immunohistochemical experiments. For the immunohistochemical experiments, the tissue was rinsed in phosphate
buffer and embedded in gelatin (gelatin type A from porcine skin; 100 g/l). The tissue was then cryofixed in N2-cooled isopentane. Sections (6 μm thick) were cut and mounted on glass slides coated with gelatin containing chromate alun [chromium(III) potassium sulfate; 0.5 g/l]. For immunofluorescence staining, after rehydration in phosphate buffer, the sections were incubated for 2 h in phosphate buffer containing 2 ml/l Triton-X-100, 30 g/l BSA, and 100 ml/l goat serum (Chemicon, Temecula, CA), to block unspecific binding sites. The blocking solution was then removed, and the sections were incubated with the primary antibody (see Table 1 for dilutions) for 24 h at 4°C. The antibodies used were monoclonal anti-HO-I and polyclonal anti-HO-II (both from StressGen, Ann Arbor, MI).

Each primary antibody was dissolved in phosphate buffer containing 1 ml/l Triton X-100, 5 g/l milk powder, 10 g/l BSA, and 10 ml/l goat serum. After they were rinsed with phosphate buffer, the sections were incubated with the secondary antibody, i.e., Cy3-labeled goat-anti-mouse or Cy3-labeled donkey-anti-rabbit (both from Dianova, Hamburg, Germany) for 1 h at room temperature. After a further rinse with phosphate buffer, the sections were incubated for 5 min with 3 · 10^{-7} mol/l 4’,6-diamidino-2-phenylindole dilactate (Molecular Probes, Leiden, The Netherlands). As a negative control, some sections were incubated with a solution that did not contain the primary antibodies (see e.g., Fig. 6, bottom row).

Reverse transcriptase polymerase chain reaction experiments. For reverse transcriptase-polymerase chain reaction (RT-PCR) studies, pieces of the intact colonic wall were transferred into lysis buffer (Qiagen, Heiden, Germany) and homogenized for 300 Hz. Total RNA was isolated using a mixer mill (NM301; Retsch, Haan, Germany) with a lysis buffer (Qiagen, Heiden, Germany) and reverse transcriptase-polymerase chain reaction (RT-PCR) protocols recommended by the manufacturer (Qiagen). RNA was reverse transcribed with Eppendorf cMaster RTPlus (Eppendorf, Hamburg, Germany) was used with 1.5 mmol/l MgCl2. Publendorf (Promega, Mannheim, Germany) using 20 μg oligo(dT)15 primer (Promega, Mannheim, Germany).

For the PCR reaction, Eppendorf MasterMix (Eppendorf, Hamburg, Germany) was used with 1.5 mmol/l MgCl2. Published primers (6) were used against rat HO-I (http://www.ncbi.nlm.nih.gov; accession code NM_012580; expected product length 200 bp) and -II (accession code J05405; expected product length 535 bp). Primers were obtained from Eurofins MWG Synthesis (Ebersberg, Germany). Each PCR started with a denaturation period of 45 s at 94°C, followed by an annealing phase of 45 s at 60°C, and an elongation phase of 1 min at 72°C; the whole cycle was repeated 50 times. For control of the PCR reaction glyceraldehyde-3-phosphate dehydrogenase (http://www.ncbi.nlm.nih.gov; accession code BC059110; forward 744 –763, backward 1027–1046; expected product length 303 bp) was used. The reaction product was visualized after electrophoresis in an agarose gel and staining with ethidium bromide.

Drugs. Bumetanide and forskolin were dissolved in ethanol (final maximal ethanol concentration 2.5 ml/l). CORM-2, cyclopinoronic acid (Alexis, Grünberg, Germany), glioblastoma (Boehringer Mannheim, Mannheim, Germany), NPPB [5-nitro-2-(3-phenylpropylamino)-benzote], ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), and YC-1 [3-(5-hydroxymethyl-2-furyl)-1-benzylindazole] were dissolved in DMSO (final maximal DMSO concentration 2.8 ml/l). Tetrodotoxin (TTX) was dissolved in 2 · 10^{-2} mol/l citrate buffer. Atropine sulfate, barium chloride, carbachol, hexamethonium, and SITS (4-acetamido-4’-isothiocyanato-stilbene-2,2’-disulfonic acid sodium salt) were dissolved in aqueous stock solutions. If not indicated differently, drugs were from Sigma (Taufkirchen, Germany). Inactivated CORM-2 was prepared as described in the literature (45).

Statistics. Results are given as means ± SE, with the number (n) of investigated tissues or cells. For the comparison of two groups, either a Student’s t-test or a Mann-Whitney U-test was applied. An F-test decided which test method had to be used. Both paired and unpaired two-tailed Student’s t-tests were applied as appropriate. To compare more than two samples, an analysis of variance was performed followed by post hoc test of Tukey. P < 0.05 was considered to be statistically significant.

RESULTS

Changes in ion transport induced by CO. The CO donor, CORM-2, evoked a concentration-dependent increase in I_{sc} across mucosa-submucosa preparations of rat colon (Fig. 1). Comparison of different colonic segments revealed that the increase in I_{sc} was more pronounced in the distal compared with the proximal colon (data not shown). Thus all experiments presented here were performed with the distal colon.

The CO donor was effective when administered either at the serosal or the mucosal side of the tissue (Fig. 1). A first significant increase in I_{sc} was evoked at a concentration of 10^{-5} mol/l at either side of the tissue. Fitting the data to a Michaelis-Menten kinetic revealed an apparent K_{m} of 2 · 10^{-5} mol/l for serosal and 10^{-4} mol/l for mucosal CORM-2. Due to this higher sensitivity against serosal CORM-2, all further experiments were performed with serosal administration of the CO donor. The residual parent compound of CORM-2 (3.5 · 10^{-4} mol/l at the serosal side) that is left after the CO is

Table 1. Antibodies used

<table>
<thead>
<tr>
<th>Target</th>
<th>Host Species</th>
<th>Supplier</th>
<th>Final Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-I</td>
<td>Mouse (monoclonal)</td>
<td>Stress Gen (OSA-111)</td>
<td>1:100</td>
</tr>
<tr>
<td>HO-II</td>
<td>Rabbit (polyclonal)</td>
<td>Stress Gen (OSA-200)</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Goat</td>
<td>Dianova (115-165-166)</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Donkey</td>
<td>Dianova (711-165-152)</td>
<td>1:800</td>
</tr>
</tbody>
</table>

HO, heme oxygenase.

Fig. 1. Concentration-dependent increase in short-circuit current (I_{sc}) across rat distal colon evoked by tricarbonyldichlororuthenium(II) dimer (CORM-2) administered either at the serosal (○) or the mucosal (□) side. Data are given as increase in I_{sc} above baseline (ΔI_{sc}) just before administration of the carbon monoxide (CO) donor and are means (symbols) ± SE (lines); n = 7.

AJP-Gastrointest Liver Physiol • VOL 300 • FEBRUARY 2011 • www.ajpgi.org
CORM-2-induced currents across the apical membrane. To more directly clarify the action sites of CORM-2 on colonic ion transport at the apical membrane, tissues were depolarized with a KCl buffer at the basolateral side. This bypasses the basolateral membrane, which is characterized by a high K⁺ conductance (11). The mucosal compartment was filled with a potassium gluconate buffer solution, so that the only ionic gradient at the apical membrane was a serosal-to-mucosal-oriented Cl⁻ concentration gradient. Consequently, all changes in $I_{sc}$ measured under these conditions reflect changes in the apical Cl⁻ conductance (40). Under these conditions, administration of CORM-2 (3.5·10⁻⁴ mol/l at the serosal side) induced a prompt increase in $I_{sc}$. Fifteen minutes after administration of the CO donor, $I_{sc}$ had increased from $-4.45 \pm 0.47$ to $-3.51 \pm 0.33 \mu$eq·h⁻¹·cm⁻² ($n = 7$, $P < 0.05$). This response was strongly inhibited when the tissues were pretreated with NPPB (10⁻⁴ mol/l at the mucosal side), a Cl⁻ channel blocker (8). In the presence of this inhibitor, the increase in $I_{sc}$ only amounted to $0.32 \pm 0.09 \mu$eq·h⁻¹·cm⁻² (Fig. 3, $n = 7$, $P < 0.05$ vs. response in the absence of NPPB).

**CORM-2 stimulates currents across the basolateral membrane.** To test whether CORM-2 has an effect on the basolateral K⁺ conductance, which determines the driving force for epithelial anion secretion, the apical membrane was permeabilized with the ionophore, nystatin (100 µg/ml). These experiments were performed in the presence of a K⁺ gradient, but in the absence of mucosal Na⁺, to avoid a contribution of the basolateral Na⁺⁻K⁺ pump to the measured $I_{sc}$ (140 mmol/l NaCl/4.5 mmol/l KCl at the serosal side, 131 mmol/l NMDG Cl⁻/13.5 mmol/l KCl at the mucosal side). Under these conditions, nystatin induces a prompt increase in $I_{sc}$, which exhibits a time-dependent spontaneous decay (34). When CORM-2 (3.5·10⁻⁴ mol/l at the serosal side) was administered, this

---

**Fig. 2.** Anionic dependency and sensitivity against transport inhibitors of the $I_{sc}$ response evoked by CORM-2 (2.5·10⁻⁴ mol/l at the serosal side). For each experimental series, the response to CORM-2 was tested in the presence of a transport inhibitor or after anion substitution (solid bars), respectively, and compared with the response under control conditions (open bars). Inhibitors used were as follows: bumetanide (10⁻⁴ mol/l at the serosal side), 4-acetamido-4'-isothiocyanato-stilbene-2,2’-disulfonic acid sodium salt (SITS; 10⁻³ mol/l at the serosal side), and glibenclamide (5·10⁻⁴ mol/l at the mucosal side). Anion substitution was performed on both the mucosal and the serosal side of the tissue. Data are given as $\Delta I_{sc}$ just before administration of the CO donor and are means (symbols) ± SE; $n = 7–10$. *$P < 0.05$ vs. response evoked by CORM-2 in the respective control series. Obviously, the control response evoked by CORM-2 (open bars) exhibited a certain degree of variability (this was the reason for performing parallel control experiments for each experimental series), although the differences in the amplitude of the individual control series did not reach statistical significance in the analysis of variances.

Released from the molecule was ineffective; it induced a change in $I_{sc}$ of 0.05 ± 0.018 µeq·h⁻¹·cm⁻² ($n = 8$; not significant).

Repeated administration of CORM-2 resulted in a desensitization of the tissue, even when the serosal compartment was washed three times with five times the chamber volume after each administration of the drug. The second administration of CORM-2 (2.5·10⁻⁴ mol/l at the serosal side) caused an increase in $I_{sc}$ of only 6.6 ± 1.2% ($n = 11$). Therefore, CORM-2 was administered only once to an individual tissue in all further experiments. Tissue conductance after administration of CORM-2 (2.5·10⁻⁴ mol/l at the serosal side) increased by 4.6 ± 1.9 mS/cm² ($n = 7$).

**Ionic nature of the $I_{sc}$ response.** After inhibition of the basolateral Na⁺⁻K⁺⁻Cl⁻ cotransporter with bumetanide (10⁻⁴ mol/l at the serosal side), the effect of CORM-2 on $I_{sc}$ was only marginally reduced (Fig. 2). Similarly, serosal application of the stilbene derivative SITS (10⁻³ mol/l at the serosal side), which inhibits the basolateral Cl⁻/HCO₃⁻ exchanger (36), did not significantly reduce the increase in $I_{sc}$ evoked by CORM-2. However, when both blockers were combined, the CORM-2 response was significantly inhibited. An inhibition of similar size was observed when the tissues were pretreated with glibenclamide (5·10⁻⁴ mol/l at the mucosal side), an inhibitor of the CFTR (cystic fibrosis transmembrane regulator) channel (16), i.e., the dominant anion channel in the apical membrane (Fig. 2). These inhibitor experiments suggest that a secretion both of Cl⁻ and of HCO₃⁻ across apical anion channels may contribute to the effect of CORM-2 on $I_{sc}$.

This conclusion was supported by anion substitution experiments. Substitution of either Cl⁻ or HCO₃⁻ did not significantly inhibit the $I_{sc}$ induced by CORM-2. However, when both permeant anions were replaced by impermeant anions, the increase in $I_{sc}$ evoked by CORM-2 was abolished (Fig. 2).
decay (extrapolated by linear regression, Fig. 4A) was interrupted by a transient increase in $I_{sc}$ (Fig. 4A).

When the tissues were pretreated with $\text{Ba}^{2+}$ (10$^{-2}$ mol/l at the serosal side), this extrapolated increase in $I_{sc}$ was reduced from 1.00 ± 0.41 to 0.51 ± 0.12 μeq·h$^{-1}$·cm$^{-2}$, an inhibition, which did not, however, reach statistical significance (Fig. 4B).

**Involvement of secretomotor neurons in the CORM-2 effect.** Enteric neurons are able to express the two enzymes HO-I and iNOS (26). To investigate a possible involvement of secretomotor neurons in the CORM-2-induced $I_{sc}$, the serosal side was mimicked by tetrodotoxin (TTX). In the presence of this neurotoxin (10$^{-5}$ mol/l at the serosal side), the CORM-2-induced $I_{sc}$ was significantly reduced (Table 2).

Neither an inhibition of muscarinic acetylcholine receptors with atropine (5·10$^{-5}$ mol/l at the serosal side) nor an inhibition of nicotinic receptors with hexamethonium (10$^{-5}$ mol/l at the serosal side) mimicked the effects of TTX, suggesting a partial mediation of the CORM-2 response by noncholinergic enteric neurons.

**Signaling cascade.** In some tissues, such as vasculature and olfactory epithelium, CO acts via stimulating intracellular cGMP production (21, 25). However, pretreatment of the colonic preparations with ODQ (2·10$^{-5}$ mol/l at the serosal and the mucosal side), an inhibitor of the soluble guanylate cyclase (13), did not affect the increase in $I_{sc}$ evoked by the subsequent administration of CORM-2 (3.5·10$^{-4}$ mol/l at the serosal side; Table 2). Furthermore, when this pathway was pre-stimulated with YC-1 (10$^{-5}$ mol/l at the serosal and the mucosal side), an NO-independent activator of soluble guanylate cyclase (53), the response evoked by subsequently administered CORM-2 (3.5·10$^{-4}$ mol/l at the serosal side), was unaltered (Table 2). Hence, there must be another pathway for CO in rat colonic epithelium.

**Changes in the cytosolic $Ca^{2+}$ concentration evoked by CORM-2.** One intracellular second messenger with a profound effect on colonic ion transport is $Ca^{2+}$. To find out whether CORM-2 might induce a change in the cytosolic $Ca^{2+}$ concentration, isolated colonic crypts were loaded with the $Ca^{2+}$-sensitive fluorescent dye, fura-2. In these crypts, CORM-2 (2·10$^{-4}$ mol/l) induced a prompt increase in the fura-2 ratio signal, i.e., an increase in the cytosolic $Ca^{2+}$ concentration (Fig. 5, top right), which was not observed in the time-dependent control experiments (Fig. 5, top left). On average, the fura-2 ratio increased from 1.16 ± 0.06 to 1.80 ± 0.12 ($n =$ 70, $P < 0.05$) 30 min after administration of the CO donor.

Obviously, a release of $Ca^{2+}$ from intracellular stores is not involved in this response, as the effect of CORM-2 was unaltered when the crypts were pretreated with cyclopiazonic acid (5·10$^{-5}$ mol/l), an inhibitor of sarcoplasmic-endoplasmic $Ca^{2+}$ ATPases. In the presence of this inhibitor, CORM-2 still evoked an increase of the fura-2 ratio signal from 1.41 ± 0.08
pretreatment of the tissue with CORM-2 and of 10.06\% H9262

gogue carbachol (5·10^-4 mol/l) was still preserved. In contrast, the Ca2+
impatience to study its interaction with typical Ca2+- or cAMP-dependent secretagogues. When tissues had been exposed once to CORM-2 (3.5·10^-4 mol/l at the serosal side without intermediate washing procedures), the activator of the adenylyl cyclase, forskolin (5·10^-6 mol/l at the mucosal and the serosal side, administered ~30 min after CORM-2) evoked an increase in Isc of 3.32 ± 1.01 \mu eq·h^{-1}·cm^{-2} above baseline (n = 13). In time-dependent control experiments, which had not been pretreated with CORM-2, forskolin evoked an increase in Ic of 5.48 ± 1.46 \mu eq·h^{-1}·cm^{-2} above baseline (n = 12; difference not significant vs. response in the presence of CORM-2), i.e., the secretory capacity of the tissue evoked by the cAMP pathway was still preserved. In contrast, the Ca2+-dependent secretagogue carbachol (5·10^{-5} mol/l at the serosal side) induced an increase in Ic of 3.61 ± 0.33 \mu eq·h^{-1}·cm^{-2} (n = 7) after pretreatment of the tissue with CORM-2 and of 10.06 ± 1.27 \mu eq·h^{-1}·cm^{-2} (n = 7) without CORM-2 pretreatment (P < 0.05 vs. response to carbachol in the presence of CORM-2).

Bearing in mind that CORM-2 evokes an increase in the cytosolic Ca2+ concentration (Fig. 5), this apparent inhibition of the carbachol response is not unexpected considering the well-known downregulation of the Ca2+-signaling pathway after repeated administration of Ca2+-dependent secretagogues by still incompletely known mechanisms (see e.g., Ref. 35).

Detection of HO-I and -II. To clarify whether the colonic wall might be able to produce CO, immunohistochemical experiments were performed with antibodies against the two enzymes, HO-I and -II, which are responsible for endogenous CO production. Immunoreactivity of HO-I was found mainly in the muscularis propria (Fig. 6). In contrast, HO-II was localized in addition within the colonic epithelium (Fig. 7).

These results were confirmed by RT-PCR. Amplificates for HO-I (target size of 200 bp), HO-II (target size of 554 bp), and the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase were found in samples from the intact wall of rat distal colon (Fig. 8).

**DISCUSSION**

The present results demonstrate that the CO donor CORM-2 induces a concentration-dependent increase in Isc, a measure of net charge movement, across rat distal colon (Fig. 1). A maximal response was achieved at a concentration of 2.5·10^{-4} mol/l. The total efficiency of CORM-2 to deliver CO is assumed to be 0.7 mol CO/mol CORM-2 (31). Local or plasma concentrations of free CO in vivo are not easily obtained due to the high affinity of CO to bind to heme. For example hemoglobin measurements in smokers (46) showed that, even in normal subjects, 0.4–0.7% of the 8–11 mmol/l hemoglobin in the blood is present in the form of carboxyhemoglobin (42). Consequently, a comparison with local concentrations of CO within the colonic wall in vivo is not possible. Nevertheless, the estimated resulting concentrations of CO released by CORM-2 in the present experiments are in a similar concentration range as those noted in studies in which the effect of CO delivered directly as gas was measured. For example, a concentration of 3·10^{-5} mol/l CO was found to increase the open probability of Ca2+-dependent K+ channels in vascular smooth muscle cells (51), and a maximal dilatation of rabbit thoracic aorta was achieved with CO in a concentration range of 2·4·10^{-4} mol/l (12).

A secretion of anions underlies the CORM-2-induced increase in Isc. Obviously both Cl− and HCO3− contribute to the CORM-2-induced current. Removal of only one of these permeant anions marginally affected the CORM-2-induced increase in Isc (Fig. 2). However, when both Cl− and HCO3− were substituted by impermeant anions, the response to CORM-2 was abolished. This is, however, not unexpected, because the CFTR anion channel, i.e., the dominant anion channel in the apical membrane, is permeable both for Cl−, as well as for HCO3− (16). Inhibition of this anion channel with glibenclamide significantly decreased the effect of CO in the Ussing chamber experiments (Fig. 2). Also, the experiments carried out with blockers for basolateral anion uptake transporters point to a mutual contribution of Cl− and HCO3− to the Isc.
response induced by the CO donor. Neither bumetanide, an inhibitor of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, i.e., the dominant Cl\(^-\) loading mechanism in the basolateral membrane, nor SITS, a blocker of the Cl\(^-\)/HCO\(_3\^-\) exchanger in the same membrane (36), exerted any significant inhibitory action on the CO-induced \(I_{sc}\) when applied alone. However, the combination of both inhibitors significantly reduced the response to the CO donor (Fig. 2).

One of the action sites of the CO donor seems to be the Cl\(^-\) conductance of the apical membrane. Elevation of the mucosal K\(^+\) concentration electrically eliminates the basolateral membrane with its high K\(^+\) conductance, thereby allowing the direct measurement of currents across the apical membrane (11, 34). In the presence of a serosal-to-mucosal Cl\(^-\)/HCO\(_3\^-\) concentration gradient, which served as a driving force, CORM-2 stimulated the Cl\(^-\) current across the apical membrane. This response was suppressed by NPPB, an anion channel blocker (8). Thus the increase in \(I_{sc}\) evoked by CORM-2 is carried by anion secretion across apical anion channels, mediating an efflux of Cl\(^-\) and HCO\(_3\^-\).

Several signaling pathways are known by which CO can exert its effects at different organs (14). The best documented mechanism of action is based on the similarity of CO to another gasotransmitter, NO. Effects of NO are often related to an activation of the enzyme soluble guanylate cyclase producing the second-messenger cGMP (23). In rat colon, NO causes a strong Cl\(^-\) secretion via opening of apical Cl\(^-\) and basolateral K\(^+\) channels, as well as stimulation of the Na\(^+\)-K\(^+\) pump (39). CO is known to stimulate the soluble guanylate cyclase and thereby to enhance the intracellular production of cGMP in vascular smooth muscle cells isolated from segments of rat tail arteries, leading to vasorelaxation (50). Similarly, CO increases cGMP levels in primary cultures of rat aortic vascular smooth muscle cells (29, 30, 33) and primary olfactory neurons, as well as neurons within the olfactory bulb of rats (21, 48). In human stomach and jejunum, a colocalization of HO-II and neuronal NO synthase was detected. This observation led to the suggestion of an interaction between CO and NO, the products of these two enzymes (28). Although the stimulation of cellular cGMP production by CO has been shown unequivocally, at the level of the isolated enzyme, NO is at least 30-fold more potent than CO to activate the soluble guanylate cyclase (43).

![Fig. 6. Immunohistochemical detection of heme oxygenase I (HO-I). The orientation of each picture is as follows. Top part: muscularis propria. Bottom part: surface region of the colonic epithelium. Left column: nuclear staining with 4,6-diamidino-2-phenylindole (DAPI; blue). Middle column: HO-I signal (Cy3-labeled, red). Right column: overlay of both. Top row: labeling. Bottom row: negative control without primary antibody against HO-I.](http://ajpgi.physiology.org/)

![Fig. 7. Immunohistochemical detection of HO-II. The orientation of each picture is as follows. Top part: muscularis propria. Bottom part: surface region of the colonic epithelium. Left column: nuclear staining with DAPI (blue). Middle column: HO-II signal (Cy3-labeled, red). Right column: overlay of both. Top row: labeling. Bottom row: negative control without primary antibody against HO-II.](http://ajpgi.physiology.org/)
In several tissues, the action of CO is mediated by the activation of K⁺ channels. For example, in arterial smooth muscle cells obtained from the rat tail, CO induces a relaxation via stimulation of soluble guanylate cyclase, as well as a direct activation of large-conductance Ca²⁺-activated K⁺ channels (50, 51). Similarly, CO obviously plays a role in stimulating an apical intermediate-conductance K⁺ channel in rat kidney (24).

A new mechanism of action has been proposed for the effects of CO in the alveolar epithelium (1), where CO donors inhibit Na⁺ absorption by blocking the apical epithelial Na⁺ channel. This inhibition was resistant against blockers of the cGMP signaling cascade, but was strongly reduced in the presence of the histidine-modifying agent diethyl pyrocarbonate, suggesting that CO may interact with histidine residues that are part of ion channels.

The present results suggest that the anion secretion induced by CO at rat distal colon is independent from the soluble guanylate cyclase/cGMP signaling pathway. Neither ODQ, an inhibitor of the soluble guanylate cyclase (13), nor YC-1, a NO-independent stimulator of this enzyme (53), had any effect on the CO-induced Iₛₑ (Table 2). This is in contrast to the secretory response evoked by CORM-2 at Caco-2 cells, which is strongly reduced by ODQ (47), suggesting either species differences in CO-induced signaling between human and rat or differences between native tissue and this colonic tumor cell line.

However, another second messenger, Ca²⁺, seems to play a central, previously unnoticed role in the CO response in rat colon (Fig. 5). Neurotransmitters, such as acetylcholine, or paracrine substances, such as histamine, are able to induce a Ca²⁺-dependent secretion via an elevation of the cytosolic Ca²⁺ concentration. The increased cytosolic Ca²⁺ concentration activates basolateral and apical K⁺ channels (34, 38). The consequence is a hyperpolarization of the membrane, which increases the driving force for Cl⁻ exit via apical Cl⁻ channels (3, 5, 18, 44). In the present study, imaging experiments demonstrated an increase of the cytosolic Ca²⁺ concentration induced by CORM-2 at isolated colonic crypts (Fig. 5). There were no differences in the reaction between the cells along the crypt axis, i.e., the response was independent from the degree of cellular differentiation. The increase in the cytosolic Ca²⁺ concentration was dependent on an influx of extracellular Ca²⁺ and not on a release of Ca²⁺ from intracellular Ca²⁺ stores. The pathway by which this Ca²⁺ influx occurs remains to be determined. Possible candidates are the capacitive Ca²⁺ influx, which is mediated via nonselective cation channels in these cells (10), or the Na⁺/Ca²⁺ exchanger, which can act as a Ca²⁺-loading mechanism when working in the reverse mode (41).

An increase in the cytosolic Ca²⁺ concentration is well known to activate basolateral Ca²⁺-dependent K⁺ channels (5), one of the dominant K⁺ conductances within the colonic epithelium. When the apical membrane was electrically eliminated via permeabilization with the ionophore, nystatin, CORM-2 stimulated a K⁺ current across the basolateral membrane (Fig. 4). Ba²⁺, an inhibitor of basolateral K⁺ channels, reduced this response, although this inhibition did not reach statistical significance.

Besides the basolateral membrane, which determines the driving force for anion secretion by setting the main component of the membrane potential, a further site of action of CORM-2 seems to be the apical membrane. When the basolateral membrane with its high K⁺ conductance is electrically eliminated by depolarization with a high K⁺ concentration, transepithelial Iₛₑ is determined by electrogenic ion transport across the apical membrane (11, 34). In the presence of a serosal-to-mucosal Cl⁻ concentration gradient (as driving force), CORM-2 stimulated a Cl⁻ current across this membrane, which was suppressed by a typical Cl⁻ channel blocker, such as NPPB (Fig. 3). It remains to be determined whether this response represents the activation of the dominant Cl⁻ channel in the apical membrane, i.e., the CFTR channel (16), or whether it is related to the stimulation of Ca²⁺-dependent Cl⁻ channels in the apical membrane (20). The physiological contribution of this conductance to colonic Cl⁻ secretion, compared with the contribution of the CFTR channel, is probably quite small, because Ca²⁺-induced Cl⁻ currents can only be measured when the predominant action of cytosolic Ca²⁺ on Ca²⁺-dependent K⁺ currents is suppressed by the use of high K⁺ solutions (40), but, interestingly, the activation of this conductance is blocked by an inhibitor of NO synthases (20).

Consequently, exogenous CO, released from a chemical CO donor, has the ability to modify ion transport across the epithelium of the distal colon. This raises the question of whether CO might act as modulator of epithelial functions under physiological conditions. Enteric neurons, which regulate nearly all functions of the gastrointestinal tract, express the enzymes for CO production. For example, HO-II is found in the human myenteric plexus (28), as well as in the myenteric and submucosal plexus of rat ileum (9). In neurons, the activity of HO-II is thought to be coupled to the cytosolic Ca²⁺ concentration, which increases during neuronal excitation, via activation of protein kinase C, activation of casein kinase 2, and subsequent phosphorylation of HO-II at a serine residue (4). Thus neuronally released CO might well affect epithelial transport processes. Pretreatment with the neurotoxin TTX caused a significant inhibition of the CORM-2-induced Iₛₑ (Table 2). Since this inhibition was mimicked neither by atropine, an inhibitor of muscarinic acetylcholine receptors, nor by hexamethonium, a nicotinic antagonist, this observation suggests that CO activated noncholinergic secretomotor neurons. So enteric neurons may not only be producers of CO, but might, in addition, also represent a target for this gasotransmitter.

Also, other structures within the colonic wall are equipped with the enzymatic machinery for endogenous production of CO. Immunohistochemical investigations demonstrated the existence of both enzymes responsible for endogenous CO pro-
duction in rat distal colon. HO-II is expressed over the whole tissue (Fig. 7), whereas HO-I was localized mainly in the muscularis propria (Fig. 6). These results could be confirmed by RT-PCR experiments, which detected the mRNA for both proteins within the colonic wall (Fig. 8). This raises the question of whether a basal production of CO by these enzymes might affect basal anion secretion across rat distal colon. To try to answer this question, we used zine protoporphyrine IX (10–5–10–4 mol/l at the serosal side), an inhibitor of HO-2 (15). However, this substance did not induce the expected fall in IM, but instead evoked a pronounced increase in IM (data not shown), which might suggest nonspecific actions of this inhibitor. Therefore, it remains to be determined whether endogenous CO is involved in the spontaneous anion secretion across rat distal colon in vitro or not.

Taken together, these results demonstrate that the gasotransmitter CO induces the secretion of Cl– and HCO3– across rat distal colon, a response that is dependent on the basolateral Na+/K+2Cl– cotransporter, Cl–/HCO3– exchanger, and K+ channels, as well as apical anion channels. Intracellularly, CO causes an increase in the cytosolic Ca2+ concentration via an influx of extracellular Ca2+. The expression of HO-I and HO-II within the colonic wall suggests that CO might act as a physiological modulator of colonic ion transport.

GRANTS

This study was supported by Deutsche Forschungsgemeinschaft Grant Di 388/11-1.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


