Mechanism of internal anal sphincter relaxation by CORM-1, authentic CO, and NANC nerve stimulation

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Rattan, Satish, Rany Al Haj, and Márcio A. F. De Godoy. Mechanism of internal anal sphincter relaxation by CORM-1, authentic CO, and NANC nerve stimulation. Am J Physiol Gastrointest Liver Physiol 287: G605–G611, 2004; 10.1152/ajpgi.00070.2004.—The present studies compared the effects of CO-releasing molecule (CORM-1), authentic CO, and nonadrenergic noncholinergic (NANC) nerve stimulation in the internal anal sphincter (IAS). Functional in vitro experiments and Western blot studies were conducted in rat IAS smooth muscle. We examined the effects of CORM-1 (50–600 μM) and authentic CO (5–100 μM) and NANC nerve stimulation by electrical field stimulation (EFS; 0.5–20 Hz, 0.5-ms pulse, 12 V, 4-s train). The experiments were repeated after preincubation of the tissues with the neurotoxin TTX, the guanylate cyclase inhibitor 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ), the selective heme oxygenase (HO) inhibitor tin protoporphyrin IX (SnPP-IX), the nitric oxide synthase inhibitor Nω-nitro-arginine (L-NNA), and SnPP-IX + L-NNA. We also investigated the effects of the HO substrate hematin (100 μM). CORM-1, as well as CO, produced concentration-dependent IAS relaxation, whereas hematin had no effect. TTX abolished and L-NNA significantly blocked IAS relaxation by EFS without any effect on CORM-1 and CO. ODQ blocked IAS relaxation by CORM-1, authentic CO, and EFS. SnPP-IX had no significant effect on IAS relaxation by CORM-1, CO, or EFS. The presence of neuronal nitric oxide synthase, HO-1, and HO-2 in IAS smooth muscle was confirmed by Western blot studies. CORM-1 and CO, as well as NANC nerve stimulation, produced IAS relaxation via guanylate cyclase/cGMP-dependent protein kinase activation. The advent of CORM-1 with potent effects in the IAS has significant implications in anorectal motility disorders with regard to pathophysiology and potential treatments.

heme oxygenase; inhibitory neurotransmitter; nitric oxide synthase; guanylate cyclase; smooth muscle

Carbon monoxide (CO), similar to nitric oxide (NO), is acknowledged as a gaseous neurotransmitter in certain systems (3, 21). In the gastrointestinal (GI) tract, including the internal anal sphincter (IAS), it has been suggested that CO plays a role in inhibitory neurotransmission (11, 15, 23, 31, 33, 43). Similar to NO, CO is believed to act via activation of soluble guanylate cyclase (GC) (3, 21). Endogenous production of CO is one of the results of heme catalysis by heme oxygenase (HO) (3, 20, 21). In contrast to CO, the role of NO synthase (NOS) and NO in nonadrenergic noncholinergic (NANC) nerve stimulation in the GI tract has been well established (18, 32, 34, 37).

Other than suggestions from different laboratories (8, 31, 33, 43), the role of the HO pathway in NANC relaxation of GI smooth muscle has not been established (6). One of the hurdles has been the availability of a CO donor, such as NO. Administration of CO has a number of pitfalls, such as variability in the preparation and rough estimates of CO concentration in the solution. Recently, the availability of CO-releasing molecules (CORMs), such as the tricarbonyl dichlororuthenium (II) dimer [Ru(CO)2Cl2]2 (CORM-1), has made it possible to precisely examine the effects of CO in the tissues (24). The authors have shown reproducible relaxation of rat aortic smooth muscle. The effects and the mechanism of action of these interesting molecules in the GI smooth muscle are not known.

We used a three-pronged approach to examine the effects of CO in IAS smooth muscle: application of CORM-1, authentic CO, and hematin. Hematin, an HO substrate, is known to produce CO, which is responsible for certain physiological actions (17). The purpose of the present investigation was to examine and compare the effects and mechanism of action of these agents in the IAS. In addition, we compared the effects of these substances with the effects of NANC nerve stimulation while determining the role of CO and the HO pathway in NANC relaxation of the rat IAS. The studies were carried out in the rat IAS, because this animal has been recently considered to be a good model for humans (13, 40).

Materials and Methods

Tissue Preparation

Male Sprague-Dawley rats (300–350 g) were killed by decapitation, and the entire anal canal was quickly removed and transferred to oxygenated (95% O2-5% CO2) Krebs physiological solution (in mM: 118.07 NaCl, 4.69 KCl, 2.52 CaCl2, 1.16 MgSO4, 1.01 NaH2PO4, 25 NaHCO3, and 11.10 glucose) at 37°C. Extraneous adventitia, blood vessels, and skeletal muscle tissues connected to the IAS were removed using sharp dissection. The anal canal was then opened and pinned flat with the mucosal side up on a dissecting tray containing oxygenated Krebs physiological solution. Toward oxygenated Krebs physiological solution. The mucosa was removed using sharp dissection. Circular smooth muscle strips (∼0.5 × 7 mm) of the IAS (identified as a thickened circular smooth muscle situated at the lowermost part of the alimentary tract) were prepared. The experimental protocol of the study was approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and was in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Measurement of Isometric Tension

The smooth muscle strips were transferred to 2-ml muscle baths containing oxygenated Krebs solution at 37°C. One end of the muscle strip was anchored at the bottom of the tissue bath, and the other was...
connected to a force transducer (model FT03, Grass Instruments, Quincy, MA). Isometric tension was measured by the PowerLab/8SP data acquisition system (ADInstruments) and recorded using Chart 4.1.2 (ADInstruments). Each smooth muscle strip was initially stretched to a tension of 0.7 g and then allowed to equilibrate for 90 min. During this period, the smooth muscle bath was replenished with fresh Krebs solution every 20 min. Only the smooth muscle strips that developed spontaneous tone and relaxed in response to electrical field stimulation (EFS, 0.5–20 Hz, 0.5-ms pulse, 12 V, 4-s train) were used. In the presence of atropine (1 × 10⁻⁶ M) and guanethidine (1 × 10⁻³ M), EFS causes stimulation of NANC nerves in the IAS. The changes in basal IAS tone after different agents were expressed as percent maximal relaxation by EDTA (50 mM) at the end of each experiment (1, 5).

**Preparation of CORM-I, CO, and Other Agents**

CORM-I was freshly prepared before each experiment. A stock solution of 10⁻³ M was obtained by dissolving CORM-I in DMSO following previously published instructions (24). Aliquots of this solution were then delivered to the tissues as described earlier (24) to obtain the final desired concentrations in the muscle bath.

CO was prepared following the method described by Schröder et al. (35). Briefly, 20 ml of Krebs solution were deoxygenated for 1 h with helium gas in a sealed glass vial. The solution was then bubbled with 99.8% CO for 15 min until a saturated (10⁻³ M) solution was obtained. Tin protoporphyrin IX (SnPP-IX) was dissolved in 0.2 N NaOH and hematin in 0.1 N NaOH. N⁵-nitro-l-arginine (l-NNA) was dissolved in distilled water, and 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one (ODQ) was dissolved in DMSO. The final concentration of DMSO in the organ bath did not exceed 0.1%.

**Drug Responses**

Concentration-response curves with 50–600 µM CORM-I and 5–100 µM CO were obtained in a cumulative fashion as described elsewhere (25). The O₂ supply to the muscle bath was temporarily turned off briefly during such studies. The temporary cessation of oxygenation had no significant effect on basal IAS tone. To investigate the mechanism of action of these molecules, these experiments were repeated 20 min after incubation with different inhibitors: the neurotoxin TTX (1 × 10⁻⁶ M), the selective HO inhibitor SnPP-IX (1 × 10⁻⁶ M), the NOS inhibitor l-NNA (3 × 10⁻⁴ M), and the GC inhibitor ODQ (1 × 10⁻⁶ M). NANC nerve stimulation experiments by EFS were done in the presence of guanethidine (1 × 10⁻³ M) and atropine (1 × 10⁻⁶ M).

**Western Blot Analysis**

The presence of HO-1 and HO-2 and neuronal NOS (nNOS) was determined by Western blot studies as described elsewhere (7, 8, 12). In the presence of atropine (1 × 10⁻⁶ M) and guanethidine (1 × 10⁻³ M), EFS causes stimulation of NANC nerves in the IAS. The changes in basal IAS tone after different agents were expressed as percent maximal relaxation by EDTA (50 mM) at the end of each experiment (1, 5).

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**Western Blot Analysis**

The presence of HO-1 and HO-2 and neuronal NOS (nNOS) was determined by Western blot studies as described elsewhere (7, 8, 12). Iso-β-actin expression was used as standard for calculations. Briefly, the smooth muscle tissues were cut in small pieces (~1-mm cubes), rapidly homogenized in five volumes of boiling lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris, pH 7.4), and then microwaved for 10 s. The homogenates were centrifuged (16,000 g at 4°C) for 15 min, and protein contents in the resultant supernatant were determined by the method of Lowry et al. (19) with BSA as the standard. The samples were then mixed with 2× sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromphenol blue, and 2% β-mercaptoethanol) and placed in a boiling water bath for 3 min. The proteins in an aliquot (20 µl containing 40 µg of protein extract) of each sample were separated by 7.5% SDS-polyacrylamide gel. The proteins thus separated were transferred to a nitrocellulose membrane (NCM) by electrophoresis at 4°C. To block nonspecific antibody binding of the antibodies, the NCMS were soaked overnight at 4°C in Tris-buffered saline-Tween 20 (TBST; 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) containing 1% BSA. NCMS were then incubated with the specific primary antibodies [goat polyclonal IgG (1:2,000) for HO-1 and HO-2, rabbit polyclonal IgG (1:2,000) for nNOS, and β-actin] for 1 h at room temperature. After they were washed with TBST, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-goat IgG secondary antibodies (1:25,000) for detection of HO-1 and HO-2 and peroxidase-conjugated secondary anti-rabbit IgG secondary antibodies (1:25,000) for detection of nNOS and β-actin for 1 h at room temperature. The corresponding bands were visualized with enhanced chemiluminescence substrate using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and Hyperfilm MP (Amer sham Life Science).

NCMs were then stripped of primary and secondary antibodies by incubation with Restore Western blot stripping buffer (Pierce) for 15 min at room temperature. NCMs were soaked overnight at 4°C in TBST. Immunoblots for β-actin were obtained using primary and secondary antibodies as described above. Bands corresponding to different proteins on X-ray films were scanned (SnapScn.310, Agfa, Ridgefield Park, NJ), and the respective areas and optical densities were determined using Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

**Data Analysis**

Values are means ± SE of different observations. Agonist concentration-response curves were fitted using a nonlinear interactive fitting program (Prism 3; Graph Pad Software). Agonist potencies and maximum response are expressed as the negative logarithm of the molar concentration of agonist producing 50% of the maximum response and the maximum effect elicited by the agonist, respectively, calculated from the concentration-response curves. Statistical significance was determined by one-way ANOVA or Student’s t-test where suitable. In all cases, P < 0.05 was used to determine statistical significance.

**RESULTS**

**Effect of CORM-I vs. CO on Basal Tone of IAS**

Influence of different neurohumoral antagonists and the neurotoxin TTX. CORM-I, as well as CO, produced concentration-dependent relaxation in the rat IAS. None of the neurohumoral antagonists (hexamethonium, propanolol, guanethidine, atropine, and indomethacin) had a significant effect on the relaxant actions of CORM-I or CO (data not shown). The neurotoxin TTX (1 × 10⁻⁶ M) also had no significant effect on the IAS relaxation caused by these agents (P > 0.05, n = 5–10 animals; Fig. 1, A and B). TTX, on the other hand, nearly abolished the IAS relaxation with EFS (Fig. 1C). ω-Conotoxin (1 × 10⁻⁶ M), which causes significant attenuation of EFS-induced IAS relaxation, also had no significant effect on CORM-I- and CO-induced IAS relaxation (not shown).

Influence of SnPP-IX on IAS smooth muscle contraction. The HO inhibitor SnPP-IX (1 × 10⁻⁴ M), previously shown to cause significant suppression of
NANC relaxation and HO activity in different systems (2, 8, 31, 33, 43), caused no attenuation of IAS relaxation by the maximal effective concentrations of CORM-1 and CO (*P < 0.05, n = 5–10; Fig. 2, A and B). Unexpectedly, however, 10–3 or 3 × 10–4 M SnPP-IX had no significant effect on EFS-induced relaxation in rat IAS smooth muscle (P > 0.05, n = 4–6; Fig. 2C). In the presence of 10–4 SnPP-IX, EFS at 5 and 10 Hz caused a decline of 66.4 ± 4.5 and 66.0 ± 6.3% in basal IAS tone. In control experiments, the decline in basal IAS tone was 63.6 ± 6.1 and 69.5 ± 5.0%, respectively.

**Effect of t-NNA on IAS Relaxation by CORM-1, CO, and EFS**

Similar to their effect when used individually, the NOS and HO inhibitors in combination also produced no significant effect on the decline of basal IAS tone caused by CORM-1 or CO (P > 0.05, n = 6–8; Fig. 4, A and B). In contrast, t-NNA + SnPP-IX caused a significant attenuation of the IAS relaxation caused by EFS (P < 0.05, n = 10; Fig. 4C). In the presence of t-NNA + SnPP-IX, however, the EFS-induced relaxations were not significantly different from relaxation induced by t-NNA alone (P > 0.05; Figs. 2C and 3C).

**Effect of ODQ on IAS Smooth Muscle Relaxation by CORM-1, CO, and EFS**

The GC inhibitor ODQ (10–6 M) significantly attenuated the IAS relaxation caused by CORM-1, CO, and EFS (P < 0.05, n = 3–4). There were, however, interesting differences in terms of the degree of this attenuation by ODQ.
effects of CO were nearly abolished, whereas the GC inhibitor caused quantitative antagonism of CORM-1 and a rightward shift in the EFS-induced relaxation of IAS smooth muscle. The trends in IAS relaxation with these stimuli with a higher concentration of ODQ (10^{-5} M) were similar. The maximal effective concentration of CORM-1, CO, and 10 Hz of EFS in the presence of ODQ resulted in IAS relaxation of 24.7 ± 6.9, 4.40 ± 10.26, and 27.22 ± 6.82%, respectively (P < 0.05, n = 3–4; Fig. 5).

**Effect of Hematin on Basal IAS Tone and IAS Relaxation With EFS**

To further investigate the role of the HO pathway in the rat IAS, we examined the effect of the HO substrate hematin. Unexpectedly, incubation of the tissues with 10^{-4} and 10^{-3} M hematin for up to 30 min caused no significant change in basal IAS tone or IAS relaxation with EFS (P > 0.05, n = 4; Fig. 6).

**Demonstration of HO-1, HO-2, and nNOS in the IAS**

HO-1, HO-2, and nNOS were demonstrated in IAS smooth muscle tissue by the Western blot technique (Fig. 7).

**DISCUSSION**

These studies, for the first time, report the relaxant effect of CORM-1 in the GI smooth muscle. This facilitates basic research on the role of CO and the HO pathway in the GI tract. The main findings of the study are as follows: 1) CORM-1, similar to CO, causes IAS smooth muscle relaxation by its action directly at the smooth muscle cell, and 2) IAS relaxation by CORM-1, CO, and EFS converges on the same intracellular mechanism, i.e., activation of GC. NANC relaxation in the rat IAS involves NOS, and the role of HO remains unclear.

Direct effects of CORM-1 and CO are evident from their independence from any neurohumoral interaction, because it is not modified by the neurohumoral antagonists, including HO and the NOS inhibitors SnPP-IX and L-NNA, respectively, and the neurotoxins TTX and ω-conotoxin. In addition, CORM-1 and CO cause concentration-dependent relaxation of smooth muscle cells isolated from the IAS. The direct effect of CO is in agreement with earlier data from our laboratory in the opossum IAS (33) and data reported by others (14, 26, 27, 42). This fulfills an important criterion for the candidate inhibitory neurotransmitter (16, 30). A direct relaxant effect of CORM-1
has been shown in vascular smooth muscle (24) but not in GI smooth muscle.

Although authentic CO should be an ideal agent for investigation of the HO pathway in NANC relaxation, its preparation is cumbersome, and calculations of CO concentrations may not be precise, because they are based on certain assumptions. In addition, the effects of CO may not exactly match those of endogenously released CO during NANC nerve stimulation. In the present studies, this issue became apparent during examination of the influence of the GC inhibitor. ODQ causes near obliteration of IAS relaxation by all concentrations of CO. The effects of CORM-1 and EFS, on the other hand, were quantitative, and their antagonism by ODQ was concentration and frequency dependent, respectively. The data suggest controlled release and delivery of CO by CORM-1 to the target site. These observations are similar to those obtained by Motterlini et al. (24). These investigators, working on the aortic smooth muscle, also reported that ODQ causes competitive antagonism of vasodilatation by CORM-1.

Our studies provide further data in support of a common mode of IAS relaxation by NANC stimulation and by CORM-1 and CO. The IAS relaxation caused by all these maneuvers converges on activation of GC. The GC inhibitor ODQ significantly attenuates the IAS relaxation caused by these stimuli. This notion is supported by a number of studies that show selective activation of GC responsible for the smooth muscle relaxation after application of CO and NANC nerve stimulation (21, 33, 43). In addition, we found the definitive presence of HO-2 in IAS tissues as shown by Western blot studies. These data are similar to previous results in the opossum and human IAS, where not only by the presence of HO-2 protein, but also by immunocytochemistry and laser capture microdissection-RT-PCR, has HO-2 been shown specifically in the myenteric plexus (8, 10). The exact role of HO-1 in the present and previous studies in the IAS is not clear.

In a number of GI preparations, including opossum and murine IAS and feline, porcine, and canine lower esophageal sphincter (2, 8, 22, 27, 42), the role of the HO pathway in NANC relaxation has been speculated. These speculations are based primarily on the effect of HO inhibitors on NANC nerve stimulation, immunocytochemical localizations of HO-2, and functional data from HO-2+/− mice. There is no such information for the rat IAS. In the present studies, we used the selective HO inhibitor SnPP-IX in the concentrations known to cause inhibition of HO-2 in different systems (2, 31). SnPP-IX causes no significant attenuation of NANC relaxation in the rat IAS. To rule out the issue of difference in affinity for HO in the

Fig. 5. Effect of the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinazolin-1-one (ODQ) on IAS relaxation by CORM-1 (A), CO (B), and EFS (C). ODQ causes significant antagonism of the fall in IAS tone by CORM-1 and EFS (*P < 0.05, n = 6–9 for EFS and n = 9–11 for CORM-1). ODQ abolishes the fall in IAS tone by CO (*P < 0.05, n = 9–10). In some experiments, CO in the presence of ODQ causes a small degree of contraction.

Fig. 6. Effect of hematin on basal IAS tone (A) and relaxation by EFS (B). Hematin at 1 × 10⁻⁴ and 1 × 10⁻³ M has no significant effect on basal tone or EFS-induced IAS relaxation (P > 0.05, n = 4).
rat IAS, we determined that even the higher concentration of SnPP-IX did not attenuate NANC relaxation.

One of the speculations for the lack of inhibition of NANC relaxation in the rat IAS by SnPP-IX is that HO inhibition may lead to overexpression of NOS activity, which compensates for the inhibition and masks the effect of SnPP-IX. To overcome this, we examined the effect of SnPP-IX in the presence of the NOS inhibitor on NANC relaxation. Even with this experimental protocol, we found no further attenuation of IAS relaxation.

Failure of hematin to exert any significant effect on IAS tone or NANC relaxation is also unforeseen. Hematin was expected to inhibit basal IAS tone and augment HO-related NANC relaxation via CO production, as shown in certain systems (17).

The main explanations for the lack of effect of HO inhibitor and precursor in the rat IAS are as follows. First, the HO pathway may not play a significant role in IAS relaxation. Second, HO inhibition by SnPP-IX upregulates constitutive NOS activity, which in turn compensates for the HO inhibition, with the net results being no effect on NANC relaxation. This concept is supported by studies that show a decrease in NOS activity after CO application (38) and by NOS upregulation after HO inhibitor (9, 36). Third, it is possible that in the rat IAS, under the experimental conditions, because of transport problems, the binding sites of HO are not accessible to the substrate and the HO inhibitor. The same situation has been shown to occur in the porcine gastric fundus (11), in which HO-2 is present in significant amounts in the myenteric plexus and CO meets a number of criteria for the inhibitory neurotransmitter (including smooth muscle relaxation by CO). Surprisingly, in those studies, as in our study, the HO inhibitor SnPP-IX had no significant effect on NANC relaxation and HO activity in the smooth muscle strips. The authors did, in fact, demonstrate that SnPP-IX inhibits HO-2 isolated directly from these tissues.

The present data in the rat IAS are in agreement with findings of previous studies in other species that NO plays a major role in NANC relaxation in the IAS (4, 28, 29, 32, 34, 39). The NOS inhibitor l-NNA in the appropriate concentrations causes significant attenuation of NANC relaxation in the IAS. However, in the presence of l-NNA, we still observed ~25% intact IAS relaxation, which was not further affected by an increase in the concentration of the NOS inhibitor. The role of other candidate inhibitory neurotransmitters such as VIP, pituitary adenylate cyclase-activating peptide, and ATP in residual IAS relaxation remains to be determined.

In summary, the studies identify an important inhibitory effect of CORM-1 in the IAS. The studies further show that, in the rat IAS, as in the human, opossum, feline, porcine, and rabbit IAS, a majority of the NANC relaxation is NO mediated.

This is in contrast to the murine IAS, where NANC relaxation has been shown to be mediated primarily via CO (41). In light of the direct effect of CORM-1 and CO in causing relaxation of the smooth muscle via GC, and the presence of significant levels of HO-2 in the IAS, a partial role of CO in the NANC relaxation, however, is difficult to rule out. In the rat IAS, HO may have a neuromodulatory role in NOS inhibitory transmission. Regardless of the role of CO in NANC relaxation, alternative approaches, such as CORM treatment, toward achieving IAS smooth muscle relaxation are important in terms of therapeutical potential in spastic anorectal motility disorders. In this regard, the refined molecules that accurately and safely deliver CO to the target site (in this case, the IAS smooth muscle cell) will be certainly preferable, because the targeted delivery of CO as gas may be neither feasible nor practical.

REFERENCES


GRANTS

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