Characterization of the motor inhibitory role of colonic mucosa under chemical stimulation in mice

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MARTÍN-CANO FE, CAMELLO PJ, POZO MJ. Characterization of the motor inhibitory role of colonic mucosa under chemical stimulation in mice. Am J Physiol Gastrointest Liver Physiol 306: G614–G621, 2014. First published February 13, 2014; doi:10.1152/ajpgi.00208.2013.—The main roles of the colonic mucosa are the absorption of water and electrolytes and the barrier function that preserves the integrity of the colonic wall. The mediators and mechanisms to accomplish these functions are under continuous investigation, but little attention has been paid to a possible control of colonic motility by the mucosa that would fine tune the relationship between absorption and motility. The purpose of this study was to establish the role of the mucosa in the control of induced colonic contractility. Young ICR-CD1 mice (3–5 mo old) were studied. Isometric tension transducers were used to record contractility in full-thickness (FT) and mucosa-free (MF) strips from proximal colon. Proximal FT strips showed lower KCl- and bethanechol-induced responses than MF strips. The difference was not due to mechanical artefacts since the contractile response of FT strips to electrical field stimulation was around 50% lower than in MF. The inhibitory effects of the mucosa on FT strips were mimicked by immersion of separate strips of mucosa in the organ bath but not by addition of mucosal extract, suggesting gaseous molecules as mediators of this effect. Incubation of MF strips with synthase inhibitors of nitric oxide, carbon monoxide, and hydrogen sulfide abolished the inhibition caused by addition of the mucosal strip, indicating that mucosal gasotransmitters are the mediators of these effects. This suggests that the control of colonic motility exerted by the mucosa could fine tune the balance between transit and absorption.

THE TWO MAIN FUNCTIONS OF the colonic mucosa are: water and electrolyte absorption and barrier function, which is the basis of the defense against luminal pathogens and toxins. The epithelial layer of the colon consists of a single sheet of columnar epithelial cells folded into finger-like invaginations called Lieberkühn’s crypts that contain four epithelial cell lineages: enterocytes, goblet cells, endocrine cells, and Paneth cells. In addition, there are multipotent stem cells located at the bottom of the crypts that differentiate into one of the epithelial cell types of the crypt wall collaborating in the physiological renewal of the mucosa (23). The epithelium maintains its selective barrier function through the formation of complex protein-protein networks that mechanically link adjacent cells and seal the intercellular space (5).

One of the most important mechanisms in the control of intestinal transport of water is acetylcholine (ACh) released from cholinergic nerves (2), which stimulates secretion from the crypts and inhibits absorption in the rat colon (1). It has been recently demonstrated that ACh can be released from epithelial cells that contain the machinery necessary for the synthesis of ACh to the basolateral space and after interaction with M3 muscarinic receptor ACh activates secretion (29). Epithelial-released ACh could then be considered as part of the nonneuronal cholinergic system and act as an intercellular messenger (11).

In addition to the classical neurotransmitters and hormones, it has become evident that small gaseous molecules, the so-called gasotransmitters, can regulate intestinal transport. Nitric oxide (NO), hydrogen sulfide (H2S), and carbon monoxide (CO) are the gasotransmitters released by the epithelium to control ionic transport (18), and the enzymes that synthesize them are present in mouse gastrointestinal epithelium (14, 16, 17). These molecules act also as enteral neurotransmitters that cause smooth muscle relaxation (4, 20, 27). Much less is known, however, about the physiological role of the mucosa on the colonic motility. It has been shown that mucosal application of short-chain fatty acids induces contraction of the colonic longitudinal muscle probably via an entero reflex involving local sensory and cholinergic nerves (28). This is in agreement with the release of nonneuronal ACh from rat colonic epithelial cells in response to luminal propionate in the presence of tetrodotoxin (TTX) (29). In the feline bladder, it has been described that the urothelium modulates bladder contractile function through local secretion of bioactive substances into the muscular layers adjacent to the urothelium. Thus, the strips without mucosa responded to electrical field stimulation (EFS), bethanechol, ATP, and KCl significantly greater than the strips with mucosa, which suggest an inhibitory role of the urothelium in the control of bladder contractility (13). This is in agreement with another study performed by the same group in guinea pig bladder (12). Although the urothelium differs from the colonic mucosa in its absorptive properties, both layers contact with smooth muscle layers whose activation propels contents. Results from our laboratory show that the mucosa has an excitatory role on spontaneous phasic contractions of proximal colon (Martín-Cano, Camello, and Pozo, unpublished observation) that is mediated by the inhibitory enteric nervous system. In the present study we examined the role of the colonic mucosa on chemically stimulated contractility of mouse proximal colon and characterized the mediators of mucosal effects on the colonic smooth muscle contractility. We have demonstrated, for the first time, that the colonic mucosa inhibits agonist-induced contractility through the release of mucosal gasotransmitters.

MATERIALS AND METHODS

Animals. Male and female Swiss ICR:CD-1 mice (3–5 mo old) were used in the study and supplied by the animal housing service from the University of Extremadura. Animals were housed in light (12:12-h light-dark cycle) - and temperature (20°C)-controlled rooms...
and had free access to food. Experimental protocols, including animal killing, were performed following ethical guidelines of the Ethical Committee of the University of Extremadura. Animal-handling procedures were reviewed and approved by the UEx Ethics Committee (23/2011) in adherence to the International Guiding Principles for Biomedical Research Involving Animals. After anesthesia with isoflurane, mice were killed by cervical transection, and the colon was immediately removed and placed in cold Krebs-Henseleit solution (K-HS).

Contraction recording of colonic smooth muscle strips. The whole colon was cleaned of fatty tissue and cut open longitudinally along the mesenteric border, and the proximal colon was identified. Circularly orientated strips (~5 × 10 mm) of proximal colon were cut and placed vertically in a 5-ml organ bath filled with K-HS maintained at 37°C and gassed with 95% O₂-5% CO₂. These strips contained all the layers of the colon (mucosa, submucosa with the submucosal plexus, circular muscle, myenteric plexus, longitudinal muscle, and serosa), and we will refer to them as full-thickness (FT) strips. To study the influence of the mucosa on the colonic contractility, we pinned the proximal segments as a flat sheet and pinched the mucosal surface with fine dissection forceps to carefully remove the mucosal layer (starting for the folds of the mucosa and continuing with the mucosa between folds, avoiding holes). When the mucosa was difficult to remove, small scissors were used to leave intact the underlying submucosal plexus. Next, the mucosa-free specimen was cut up into strips. Throughout the results we will refer to this preparation as mucosa-free (MF) strips.

Isometric contractions were measured using force displacement transducers digitized using a MacLab hardware unit and dedicated software (ADInstruments, Colorado Springs, CO). To check for the right load in both types of strips, we applied resting tensions of 0.25, 0.5, and 1 g to FT and MF strips, and when equilibrated we assayed the response to 100 μM bethanechol. No significant differences in contractility were observed, and a 0.5-g load was selected. Thus, the muscle strips were mounted under an initial resting tension equivalent to 0.5 g load and allowed to equilibrate for 60 min, with solution changes every 20 min. Strips displayed spontaneous contractility at the resting period. To induce myogenic contraction, strips were challenged with two different stimuli: K⁺-enriched K-HS (60 mM) to induce depolarization-mediated contraction and the muscarinic agonist bethanechol (100 μM) to mimic the effect of ACh, the main neurotransmitter in the gastrointestinal tract. In a group of FT and MF strips, intrinsic nerves were activated by EFS with a pair of external platinum ring electrodes (0.7 cm in diameter) connected to a square wave stimulator (Cibertec CS9/3BO) programmed through Scope software application from MacLab (AD Instruments). Trains of stimuli (0.3-ms duration, 0.5–25 Hz, 350-mA current strength) were delivered for 10 s at 3-min intervals to construct a frequency-response curve. EFS-induced effects were abolished by the Na⁺ channel blocker TTX, indicating that EFS is a selective stimulus for nerves.

For mucosal extract preparation, the mucosal layer of the proximal colon was carefully removed, and small epithelial sheets (8 × 8 mm) were kept in cold K-HS for a maximum of 2 h, and when needed they were incubated for 10 min in a 5-ml Usning chamber perfused with K-HS at 37°C and gassed with 95% O₂-5% CO₂. The mucosal extract was immediately used.

When the effect of the mucosa was assayed on MF strips, strips of mucosa (10 × 4 mm, approximately the size of muscular strips) were cut and tied with a silk thread to both ends. In one of them, a small weight was fixed to have the mucosa near by the muscle strip but not too close to avoid artefacts. At the end of each experiment, the mucosa was dissected from FT strips since it does not contract, and the strips, including MF ones, were dried, weighed, and measured to normalize the contractile responses. Mucosal strips, used just to assay their effects on MF strips, were not weighted or measured for normalization purposes.

**Solutions and drugs.** The K-HS contained (in mM): 113 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, and 11.5 d-glucose. This solution had a final pH of 7.55 after equilibration with 95% O₂-5% CO₂.

Drug concentrations are expressed as final bath concentrations of active species. Drugs and chemicals were obtained from the following sources: aminooxacetic acid (AOAA), bethanechol, carbon monoxide-releasing molecule-A1 (CORM-A1), hemoglobin human, N²-nitro-L-arginine methyl ester (l-NAME), DL-proparglyglycine (PGP), sodium hydrosulfide hydrate (NaNHS), sodium nitroprusside (SNP), and suramin were from Sigma Chemical (St. Louis, MO); chromium mesoporphyrin IX chloride (CrMP) was from Frontier Scientific (Logan, UT); and tetrodotoxin citrate was from Tocris Cookson (Bristol, UK). Other chemicals used were of analytical grade from Panreac (Barcelona, Spain).

**Data analysis.** Results are expressed as means ± SE of 4–12 colonic strips from 47 animals. Colonic strip tension is given in millinewtons (mN). We normalized the colonic tension data by cross-sectional area [CSA (mm²) = weight (g)/specific density × length (mm), density = 1 g/mm³]. FT strips were weighted without the mucosa to exclude its noncontractile weight in the normalization. Spontaneous contractions were measured as the area under the curve (AUC) in millinewtons per square millimeter of tissue and time (mN·mm⁻²·s⁻¹). A 60-s interval was selected for analysis during agonist stimuli. When the effects of EFS were quantified, we measured the AUC of the “off” response, which consists of a contractile response immediately after finishing the electrical stimuli. The effects of the mucosa or mucosal extract are expressed as the percentage of change, taking as 100% the AUC of the 60-s period of agonist challenge in control conditions.

Statistical differences between multiple groups were tested using either one- or two-way ANOVA followed by planned-comparisons t-test. Differences were considered significant at P < 0.05.

**RESULTS**

**Mucosa exerts inhibitory effects on myogenic and neurogenic contractions.** KCl-induced depolarization (60 mM K⁺-enriched K-HS) causes a contractile response of lower magnitude in FT than in MF strips (58% of inhibition: 1,763 ± 396 vs. 4,170 ± 845 mN·mm⁻²·s⁻¹, n = 10, P < 0.05, Fig. 1A). Similarly, the muscarinic agonist bethanechol (100 μM) evoked the same increase in isometric tension that was significantly reduced in FT compared with MF strips (74.7% of inhibition: 571 ± 301 vs. 2,262 ± 101 mN·mm⁻²·s⁻¹, n = 5, P < 0.01, Fig. 1B). It could be argued that this result was due to a barrier effect of the mucosa if this layer, although very thin in mice, diminished the diffusion of KCl or bethanechol into the muscularis tunica and as result would reduce myogenic contractility. To rule out this possibility, we performed EFS to stimulate the enteric nervous system and induce neurotransmitter release within the smooth muscle layers. As it is characteristic in the colon, EFS, at frequencies higher than 0.5 Hz, induced a relaxing response that decreased the basal tension and abolished spontaneous phasic contractions. This relaxing response lasted the 10-s period of stimulation (“on” relaxation) and was followed by a rebound contraction (off contraction) with a frequency-dependent amplitude in MF strips but not in FT strips (Fig. 1C and Table 1). It was remarkable to note that the mucosa induced a strong inhibition on EFS-induced off contractions (59 and 65% of inhibition at 10 and 20 Hz, respectively, n = 10, P < 0.05, Fig. 1D and Table 1). Note this is a specific effect of the mucosa on stimulated contractility (EFS-induced response), whereas, in agreement with previous results from our group (Martin-Cano, Camello, and Pozo, 2015), the mucosa did not significantly affect force induced by 10 mM ACh in MBP strips or in MBP strips treated with CORM-A1.
unpublished observation), mucosa enhances spontaneous contractility (Fig. 1C, inter-EFS periods).

Mucosal replacement but not mucosal extracts mimics mucosa-induced effects on FT strips. To characterize the origin of the inhibitory effects of the mucosa, KCl was undesirable because it cannot be added to the organ bath directly but as substitution of KCl-enriched K-HS by the K-HS of the organ bath, which induces recording artefacts. The use of neurogenic contractions (EFS) as stimulus also has drawbacks because it would give confounding results when blockers of neurotransmitters were used, since it would not be possible to distinguish whether the effects came from mucosal or neural inhibition. Therefore, we used bethanechol to test the effects of mucosal extracts (K-HS containing mucosal-diffusible factors, see MATERIALS AND METHODS). We challenged MF strips with bethanechol, and, after washing for at least 20 min, K-HS was substituted by a mucosal extract, and the strip was immediately stimulated with bethanechol (Fig. 2A). As observed in Fig. 2B, mucosal extracts did not have any significant effect on bethanechol-induced contraction (4,023 ± 1,017 vs. 4,070 ± 915 mN·mm⁻²·s⁻¹, n = 8). However, when the second bethanechol stimulus was applied in the presence of a mucosa strip nearby the muscle strip (Fig. 2C), a significant reduction in the AUC was observed (15%, 6,564 ± 445 vs. 5,573 ± 320 mN·mm⁻²·s⁻¹, n = 6, P < 0.05, Fig. 2D).

Inhibitory effects of the mucosa on stimulated contractility are mediated by mucosal gasotransmitters. The above experimental data suggest that the inhibitory effects of the mucosa are due to gaseous or short-lived molecules such as gaseous

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**Table 1. Mucosa decreases the neurogenic response in the mouse colon**

<table>
<thead>
<tr>
<th>Type of Strip</th>
<th>EFS Frequency, Hz</th>
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<tr>
<td></td>
<td>0.5</td>
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<tr>
<td>MF</td>
<td>250.1 ± 79.5</td>
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<tr>
<td>FT</td>
<td>70.2 ± 24.1***</td>
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Mean ± SE values of the “off” contractile response to electrical field stimulation (EFS) for all of the frequencies tested (0.5, 2, 5, 10, and 25 Hz) in both types of strips [mucosa free (MF) and full thickness (FT)]; n = 10 experiments. *P < 0.05 and ***P < 0.001, planned comparisons paired Student’s t-test.
transmitters. Because the epithelial cells of the mucosa synthesize NO (7, 26) we performed organ bath studies to determine whether NO was involved in the inhibitory effects of the mucosa. After testing bethanechol-induced contractility in MF strips in control conditions (6,975 mN·mm⁻²·s⁻¹), we repeated the stimulus in the presence of a mucosal strip, which significantly reduced the response to bethanechol (5,927 mN·mm⁻²·s⁻¹, 15% of inhibition, n = 4, P < 0.05, Fig. 3A). When bethanechol was assayed in the presence of L-NAME, no differences were found with respect to control conditions (6,975 mN·mm⁻²·s⁻¹).

**Fig. 2.** Reintroduction of the mucosa in the organ bath reduces bethanechol-induced contractility, but mucosal extracts do not have any effects. **A:** representative traces of contractile responses induced by 100 μM bethanechol in MF strips in control conditions and after the substitution of the Krebs-Henseleit solution for mucosal extract. **B:** histogram showing the mean ± SE of the response of MF strips to 100 μM bethanechol in the presence and absence of mucosal extract (n = 8). **C:** representative traces of contractile responses induced by 100 μM bethanechol in MF strips in control conditions and after the inclusion of a mucosal strip nearby the MF strip. **D:** histogram showing the mean ± SE of the response induced by 100 μM bethanechol on MF strips in the presence and absence of the mucosa strip (n = 6, *P < 0.05, paired Student’s t-test).

**Fig. 3.** Nitric oxide (NO) plays a key role in the mucosal influence over colonic contractility. **A:** mucosa-induced inhibition caused by addition of the mucosa to the organ bath is reverted by pretreatment with N⁶-nitro-L-arginine methyl ester (L-NAME). Histogram showing the response of MF strips to 100 μM bethanechol in the presence and in absence of mucosa in the organ bath in control conditions and under nonnitrergic conditions (100 μM L-NAME) (n = 4, *P < 0.05, paired Student’s t-test). **B:** L-NAME treatment increases bethanechol response in FT strips. Histogram showing the response of FT strips to 100 μM bethanechol in the presence and in the absence of 100 μM L-NAME and 1 μM tetrodotoxin (TTX) (*P < 0.05, paired Student’s t-test, n = 6).
[6,920 ± 647 mN-mm⁻²s⁻¹, n = 4, not significant (NS), Fig. 3A]. Finally, l-NAME was able to block the mucosa-induced impairment of bethanechol response (6,975 ± 468 vs. 7,135 ± 755 mN-mm⁻²s⁻¹, n = 4, NS, Fig. 3A). In a different set of experiments, FT strips were stimulated in the absence and presence of l-NAME (Fig. 3B). The treatment with the nitric oxide synthase blocker enhanced the bethanechol-induced contractile response (3,314 ± 367 vs. 4,949 ± 654 mN-mm⁻²s⁻¹, 33% of increment, n = 6, P < 0.05, Fig. 3B). Similarly, after pretreatment with the Na⁺ channel blocker TTX, there was an increase, although not statistically significant, of the bethanechol response in FT strips. Interestingly, the general scavenger of NO and CO hemoglobin (80 μg/ml) significantly increased the bethanechol-induced response on FT strips (704 ± 119 vs. 1,059 ± 194 mN-mm⁻²s⁻¹, n = 4, P < 0.05). Taken together, this set of data indicates that the mucosa provides a significant source of NO, since the blockade of the neuronal NO release by TTX was not capable of increasing the response to bethanechol significantly.

In addition to NO, it has been described that mouse gastrointestinal mucosa releases H₂S and CO (14, 16, 17). To determine the possible participation of these neurotransmitters in the inhibitory effects of the mucosa, we assayed the effects of blockers of H₂S and CO synthesis. In addition, we tested the effects of suramine, a blocker of the nongaseous inhibitory neurotransmitter ATP, as a negative control. The protocol used was the same as the one used to check the effects of l-NAME: a first challenge with bethanechol in control conditions and a second challenge in the presence of both the mucosal strip and the blocker of choice or just the mucosa in the control group. As represented in Fig. 4, the colonic mucosa decreased significantly the response to bethanechol, but not when blockers of H₂S-producing enzymes cystathionine γ-lyase (AOAA, 500 μM) and cystathionine β-synthase (PPG, 1 mM) were present. This was also the case for CrMP (5 μM), a blocker of hemoxygenase activity, or when all these blockers and L-NAME were added at the same time (all blockers group). However, the blockade of ATP neurotransmission by suramine (100 μM) did not reduce the inhibitory effects of the mucosa (n = 5, P < 0.05, see data in Table 2). These results suggest that an inhibitory gaseous neurotransmitter is mediating the inhibitory effects of the mucosa on bethanechol-induced contractility. In keeping with this, when the mucosa strip was previously incubated for 10 min in the presence of inhibitors of the synthesis of the above-mentioned gasotransmitters, the inhibitory effect was not present (7,308 ± 560 vs. 7,630 ± 414 mN-mm⁻²s⁻¹, n = 4, Fig. 5), reinforcing a possible intervention of these gaseous mediators. To rule out that the inhibition of gaseous neurotransmitters released by enteric nerves located in the muscle layers would be increasing the response to bethanechol, we checked in MF strips the effects of bethanechol in control conditions and in the presence of AOAA + PPG, l-NAME, and CrMP. The treatment did not have significant effects on bethanechol-induced contractility in MF strips (3,934 ± 508 vs. 3,041 ± 469 mN-mm⁻²s⁻¹, n = 13, NS), suggesting a mucosal source of gasotransmitters under stimulation.

To confirm that the release of gaseous transmitters would decrease bethanechol contractility in MF strips, after testing the response to bethanechol in control conditions, donors of the gasotransmitters, such as sodium nitroprussiate (100 μM), NaHS (300 μM), and CORM-A1 (160 μM), were added to the organ bath, and then bethanechol was assayed. As shown in Fig. 6, these donors significantly reduced spontaneous phasic contractions and bethanechol-induced contractility (SNP:

Table 2. Mucosa does not reduce bethanechol-induced contractility when the synthesis of gasotransmitters is blocked

<table>
<thead>
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<th>Bet (100 μM)</th>
<th>Bet (100 μM) + Mucosa</th>
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<tbody>
<tr>
<td>Control</td>
<td>3,785.7 ± 371.3</td>
<td>3,457.5 ± 384.8**</td>
</tr>
<tr>
<td>l-NAME</td>
<td>3,567.1 ± 377.9</td>
<td>4,804.1 ± 787.2</td>
</tr>
<tr>
<td>AOAA + PPG</td>
<td>3,257.9 ± 594.6</td>
<td>3,293.3 ± 679.5</td>
</tr>
<tr>
<td>CrMP</td>
<td>4,804.2 ± 787.2</td>
<td>4,709.8 ± 845.5</td>
</tr>
<tr>
<td>All blockers</td>
<td>3,041.4 ± 469.2</td>
<td>3,008.1 ± 431.4</td>
</tr>
<tr>
<td>Suramine</td>
<td>3,387.5 ± 681.6</td>
<td>3,129.7 ± 633.7*</td>
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</table>

Mean ± SE values of the bethanechol (Bet)-induced contractile response in the presence and absence of the mucosa into the organ bath. These data were obtained in control conditions and after the selective blockade of the synthesis of nitric oxide (NO) [100 μM N⁵-nitro-l-arginine methyl ester (l-NAME)], hydrogen sulfide (H₂S) [500 μM aminoacidic acid (AOAA), 1 mM DL-propargylglycine (PPG)], carbon monoxide (CO) [5 μM chromium mesoporphyrin IX chloride (CrMP)], and ATP (100 μM suramine) or the joint blockade of NO, H₂S, and CO synthesis (all blockers). Control, n = 18, **P < 0.01, paired Student’s t-test. Suramine, n = 5, *P < 0.05, paired Student’s t-test.
DISCUSSION

In the present study, we have demonstrated for the first time that the mucosal layer plays an inhibitory role on the colonic motility induced by depolarization, the muscarinic agonist bethanechol, and activation of the enteric nervous system by EFS. Inhibitory gaseous molecules released by the epithelial layers such as NO, H2S, and CO mediate this inhibition. The possibility that this inhibition was due to a mechanical effect of the mucosa that would act as a barrier for the agonists used in the study can be ruled out, since the mucosa also inhibited EFS-induced contractility. Out of the three stimuli used to induce contraction, activation of enteric nerves by EFS

![Fig. 5](image)

Fig. 5. The blockade of the synthesis of gasotransmitters abolishes the inhibitory effect of the mucosa on bethanechol-induced contractility. A: representative traces of contractile responses induced by 100 µM bethanechol in MF strips in control conditions and after the inclusion of a mucosal strip previously incubated for 10 min with inhibitors of the synthesis of gaseous neurotransmitters (100 µM L-NAME, 500 µM AOAA + 1 mM PPG, and 5 µM CrMP). B: histogram showing the mean ± SE of the response induced by 100 µM bethanechol on MF strips in the presence and absence of previously inhibited mucosa.

4,127 ± 470 vs. 2,086 ± 288 mN·mm⁻²·s⁻¹, 49% of inhibition, n = 7, P < 0.001, Fig. 6B; NaHS: 5,742 ± 1,366 vs. 2,043 ± 1,065 mN·mm⁻²·s⁻¹, 65% of inhibition, n = 5, P < 0.0, Fig. 6D; CORM-A1: 6,444 ± 775 vs. 2,332 ± 498 mN·mm⁻²·s⁻¹, 64% of inhibition, n = 8, P < 0.01, Fig. 6F).

Fig. 6. NO, H2S, and CO donors decrease the response to bethanechol. A, C, and E: original isometric contraction traces from circular-orientated MF strips showing the contractile response to 100 µM bethanechol in the presence and absence of 100 µM sodium nitroprusside (SNP), 300 µM NaHS, and 160 µM carbon monoxide-releasing molecule-A1 (CORM-A1), respectively. B: histogram showing the mean ± SE of the response of MF strips to 100 µM bethanechol in the presence and absence of 100 µM SNP (n = 7, ***P <0.001, paired Student’s t-test). D: histogram showing the mean ± SE of the response of MF strips to 100 µM bethanechol in the presence and absence of 300 µM NaHS (n = 5, *P < 0.05, paired Student’s t-test). F: histogram showing the mean ± SE of the response of MF strips to 100 µM bethanechol in the presence and absence of 160 µM CORM-A1 (n = 8, **P < 0.01, paired Student’s t-test).
is the closest approach to in vivo conditions, since neurotransmitters are released from motor nerve varicosities to the “neuroeffector” junction where interstitial cells of Cajal, enteric neurons, glial cells, and smooth muscle cells, among other cells, interact to induce contraction (19). The fact that FT strips contract in response to EFS <50% compared with MF strips clearly points out that this is a physiological role of the mucosa.

The negative influence of the mucosa on the colonic motility can only be explained by the release of relaxing substances from the mucosa. The stimulatory effect of the hemoglobin on FT strips points to a role for NO and CO as mediators of this effect. The lack of effects of the mucosal extracts and the reduction of motility evoked by the addition of a mucosa strip nearby the MF strip indicates that gaseous molecules should be the mediators. NO, H2S, and CO have been described as gaseous neurotransmitters in the enteric nervous system and also play a role in the response to gastrointestinal injury (5, 8, 15). Our result with specific inhibitors of their synthetic enzymes clearly demonstrates that the three of them are involved in the effects of the mucosa, which is reinforced for the lack of effects of mucosal strips previously treated with these inhibitors. There is evidence for the expression of the key enzymes for NO, H2S, and CO formation in gastrointestinal epithelial cells (6, 7, 25, 26), including mice (14, 16, 17). Activation of their epithelial release when the mucosa is stimulated by bethanechol would explain the release from the mucosa once it has lost its connections with the submucosal and myenteric plexus (in some of our experimental conditions). However, the inhibitory effect of the mucosa in FT strips is higher than the effect of addition of the mucosa to MF strips (compare Figs. 1 and 3A), which could be due to the functional damage of the mucosa as consequence of experimental manipulations, to the increase in the space between the mucosa and the muscularis externa, which would drastically reduce the amount of molecules reaching the muscular layer, or to the interruption of nerve connections between the mucosa and enteric plexuses. We can speculate that both mucosal and enteric nerve-induced release coexist, similar to the control of mucosal secretion by ACh of both neural and nonneural origin (3, 29). It has to be noted that a transwall resting membrane potential (RMP) gradient across the circular smooth muscle layer has been described in MF colonic tissue. This RMP gradient is dependent on release of CO from submucosal neurons (21), and it has been recently shown that endogenously generated H2S acts as a stealth hyperpolarizing factor to maintain the CO-dependent transwall gradient (22). In keeping with this, the submucosal plexus from our MF strips has an inhibitory character (when the submucosa layer is removed, the response to betanechol increases by 30%, Martín-Cano, Camello, and Pozo, unpublished observation). It is then possible that, when mucosa was stimulated, the RMP gradient will increase, inducing higher inhibition of circular smooth muscle contractility.

In keeping with the motor regulatory role of the mucosa described herein, a study from our group shows an excitatory effect of the mucosa on spontaneous phasic contractions (Martín-Cano, Camello, and Pozo, unpublished observation) that agrees with the excitatory role of the mucosa in distension-evoked peristalsis (10) and fecal pellet propulsion (24). In fact, herein both effects of the mucosa controlling the colonic motility can be observed in Fig. 1C. Undoubtedly the opposite mucosal effects on motility raise more questions than this research solves. Not least among these is the physiological relevance of the dual role of the mucosa depending on its status of activation by stretch and/or chemical colonic content. The status of mucosal activation can explain the difference between the effects of exogenous application of the gasotransmitters (Fig. 6), which reduces both spontaneous phasic activity and bethanechol-induced responses, and the inhibition exerted by the mucosa that would only release these inhibitory neurotransmitters when it was under stimulation, whereas it would have an excitatory role under resting conditions. The answer could be that mucosal-induced changes in motility are aimed to accomplish the passage of the colonic content through the colon. When the colonic content moves through the colon, short-chain fatty acids will be released and would induce an increase in nonneural ACh that promotes mucosal secretion (28), and, similar to what we have observed in this report, the mucosa will inhibit colonic motor patterns by the release of gasotransmitters that would diffuse through submucosa reaching the muscular layer (9) to delay the colonic transit and favor absorption. In the interdigestive period, the lack of mucosal stimulus would have prokinetic effects to promote colonic propulsive patterns. This is a new paradigm for mucosal control of the colonic motility.

In conclusion, our results indicate that the mucosa, when stimulated, would inhibit myogenic and/or neurogenic smooth muscle contraction as the result of gasotransmitter release (NO, CO, and H2S). More studies are needed to get clearer insights into this proposed mucosal motor control.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: F.E.M.-C. and M.J.P. performed experiments; F.E.M.-C. and P.J.C. analyzed data; F.E.M.-C., P.J.C., and M.J.P. interpreted results of experiments; F.E.M.-C. prepared figures; F.E.M.-C., P.J.C., and M.J.P. edited and revised manuscript; F.E.M.-C., P.J.C., and M.J.P. approved final version of manuscript; P.J.C. and M.J.P. conception and design of research; P.J.C. and M.J.P. drafted manuscript.

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