Regional functional specialization and inhibitory nitrergic and nonnitrergic coneurotransmission in the human esophagus


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The aims of the present study are to explore, in vitro, the myenteric mechanisms of control of human LES and EB motility and the effect of nitrergic and nonnitrergic neurotransmitters. Human circular esophageal strips were studied in organ baths and with microelectrodes. Responses following electrical field stimulation (EFS) of enteric motoneurons (EMNs) or through nicotinic acetylcholine receptors were compared in the esophageal body (EB) and in clasp and sling regions in the lower esophageal sphincter (LES). In clasp LES strips: 1) sodium nitroprusside (1 nM to 100 μM), adenosine-5′-[β-thio]diphosphate triethylamine salt (1–100 μM), and vasoactive intestinal peptide (1 nM to 1 μM) caused a relaxation; 2) 1 mM Nω-nitro-l-arginine (l-NNA) shifted the EFS “on”-relaxation to an “off”-relaxation, partly antagonized by 10 μM 2′-deoxy-Nω-methyladenosine 3′,5′-bisphosphate tetradsodium salt (MRS2179) or 10 μM α-chymotrypsin; and 3) nicotine-relaxation (100 μM) was mainly antagonized by l-NNA, and only partly by MRS2179 or α-chymotrypsin. In sling LES fibers, EFS and nicotine relaxation was abolished by l-NNA. In the EB, l-NNA blocked the latency period, and MRS2179 reduced “off”-contraction. The amplitude of cholinergic contraction decreased from the EB to both LES sides. EFS induced a monophasic inhibitory junction potential in clasp, sling, and EB fibers abolished by l-NNA. Our study shows a regional specialization to stimulation of EMNs in the human esophagus, with stronger inhibitory responses in clasp LES fibers and stronger cholinergic excitatory responses in the EB. Inhibitory responses are mainly triggered by nitrergic EMNs mediating the inhibitory junction potentials in the LES and EB, EFS on-relaxation in clasp and LES sides, and latency in the EB. We also found a minor role for purines (through P2Y1 receptors) and vasoactive intestinal peptide–mediating part of nonnitrergic clasp LES relaxation.

lower esophageal sphincter; esophageal body; inhibitory motoneurons; inhibitory coneurotransmission

THE MAIN PHYSIOLOGICAL FUNCTION of the lower esophageal sphincter (LES) is to generate a zone of high pressure that prevents the reflux of acid gastric content into the esophagus. LES relaxations occur briefly after swallowing, and transient LES relaxations cause physiological gastroesophageal reflux and allow belching. Enteric motoneurons (EMNs) are the final step in the inhibitory vagal pathway to the LES, mediating swallow-induced and transient LES relaxation (3). Nitric oxide (NO) released from these inhibitory EMNs is the major contributor to LES relaxation in humans (12) and opossums (20, 33). However, vasoactive intestinal peptide (VIP) and ATP are putative inhibitory neurotransmitters in animal studies (14, 21–24, 34, 37, 39), and the relative contribution of each of these neurotransmitters remains unclear in humans. Our laboratory has recently found that the relaxation of porcine LES is caused by two main neural pathways of similar magnitude: 1) NO through guanylate cyclase signaling pathways; and 2) an apamin-sensitive pathway mediated by ATP or a related purine acting through P2Y1 receptors (7). In contrast, we found that, in humans, NO is the main neurotransmitter released after stimulation of inhibitory EMNs and causes 85% of LES relaxation, an apamin-sensitive neurotransmitter having only a minor role (12). The role of purines and VIP in human LES relaxation has not been established.

In vivo manometric studies performed on humans and animals have shown significant radial asymmetry in the distribution of LES pressures, the highest pressures being recorded in the left lateral direction of the LES (26, 28, 29, 36). Moreover, both in humans (28) and animals (26), atropine decreases LES pressure more on the left than the right side. The macroscopic arrangement of LES inner muscle circular smooth fibers in humans and pigs is very similar, with semicircular transverse muscle clasps on the lesser curve side (“clasp fibers”) and long oblique gastric “sling” fibers in the greater curvature on the left side of LES (17, 36). Porcine LES shows a strong transversal functional asymmetry, as neural stimulation induces stronger nitrergic responses and weaker cholinergic responses in clasp fibers and, in contrast, weaker nitrergic responses and stronger cholinergic responses in the sling region (8). A very recent study on mice also found regional differences in the inhibitory junction potential (IJP) between clasp and sling LES sides (41).

In the human and porcine esophageal body (EB), the circular fibers are arranged in rings, and both inhibitory and excitatory EMNs play an important role in the peripheral mechanism of EB peristalsis. Animal studies found cholinergic nerve stimulation enhanced the amplitude and duration of esophageal peristalsis, with the influence of cholinergic EMNs being most prominent proximally along the smooth muscle portion of the esophagus (5). Human studies showed that activation of inhibitory EMNs preceded that of cholinergic EMNs during EB peristalsis (30). However, the neuromyogenic mechanisms of the regional functional specialization in the LES and the EB have not been fully characterized in humans.

The aims of the present study are to explore, in vitro, the myenteric mechanisms of control of human LES and EB mo-
ility and the effect of nitriergic and nonnitriergic inhibitory neurotransmitters in the human esophagus (LES and EB).

**METHODS**

**Preparations**

We studied specimens of the gastric fundus, the gastroesophageal junction, and the EB from 9 organ donors, and more restricted specimens from the LES and EB from 15 patients with esophageal cancer. The overall sample included 24 esophageal specimens (4 women/20 men; mean age, 53.7 ± 3.5 yr). The study protocol was approved by the Institutional Review Board of the Hospital de Marató, Barcelona, Spain. The gastroesophageal junction was opened along the anterior side, the mucosa was carefully removed, and the clasp and sling fiber bundles of LES were readily identified as a thickened band of semicircular oriented smooth muscle adjacent to the greater and lesser curvature of the stomach, respectively (17, 36). Full thickness preparations, including the circular and longitudinal muscle layers, as well as the myenteric plexus, were obtained from each LES side (clasp/sling strips) by cutting 3-mm-wide strips parallel to circular muscle fibers (7, 12). Up to four consecutive strips were obtained from both the clasp and sling region of the LES of each specimen from organ donors and from the EB, 5 cm above the transitional line (8, 12). The weight of the strips was similar in the three regions studied: 1) clasp, 0.29 ± 0.01 g (n = 18); 2) sling, 0.31 ± 0.02 g (n = 13); and 3) EB, 0.31 ± 0.02 g (n = 28).

**Solutions and Drugs**

The Krebs solution used in these experiments was the same as in our laboratory’s previous studies (7, 8, 12). Sodium nitroprusside (SNP), nicotine, nifedipine, tetrodotoxin (TTX), the selective P2X antagonist 6-methyladenosine 3',5'-cyclic monophosphate trilithium salt (ADP·N), Louis, MO). The competitive antagonist for P2Y1 receptors 2/H9251-chymotrypsin, and VIP were obtained from Sigma Chemical (St.

**Techniques**

**Organ bath studies.** Final strips measuring 10 mm in length were placed in 15-ml organ baths containing Krebs’ solution constantly bubbled with 5% CO₂ in O₂. Changes in tension of the strips were measured using isometric force transducers (model 03 Force Transducer and model 7 Series Polygraph, respectively, Grass Instruments, Quincy, MA), computed using the data acquisition software AcqKnowledge 3.7.2 (Biopac Systems). After an equilibration period of 60 min, strips were stretched up to 150% of their initial length and positioned between two parallel platinum wire electrodes 10 mm apart (7, 8, 12). Thereafter, strips taken from the LES progressively increased their tone during the following 1–2 h, and those from the EB did not. Total tone achieved by LES strips was defined as the tone developed by the strips after the equilibration period. Electrical field stimulation (EFS) was applied by means of an electrical stimulator (model S88, Grass Instruments Co) and a power booster (Stimul-Splitter II, Med-Lab Instruments, Loveland, CO) (12). These electrical stimuli were simultaneously recorded with tracings of mechanical responses through a synchronized transistor-transistor logic signal between the electrical stimulator Grass S88 and the computerized Biopac System to assess whether the mechanical responses occurred during (“on”-responses) or after (“off”-responses) the electrical stimulus. Only the strips that developed spontaneous tone during the equilibration period and relaxed during EFS were considered as pertaining to the LES.

**Electrophysiological studies.** Transmural circular LES and EB muscle strips were placed in a Sylgard-coated chamber continuously perfused with Krebs solution at 37 ± 1°C. Strips were pinned on a cross-sectioned slab, allowing microelectrode recordings of circular muscle (9). Smooth muscle cells from the circular layer were impaled with glass microelectrodes (40–60 MΩ) filled with 3 M KCl. Membrane potential was measured using standard electrometer Du0773 (WPI). Tracings were displayed on an oscilloscope 4026 (Racal-Dana, England) and simultaneously digitalized (100 Hz) using PowerLab/40 system and Chart 5 software for Windows (all from ADInstruments, Castle Hill, NSW, Australia). EFS was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. Nifedipine (1 μM) was used to abolish the mechanical activity during electrophysiological experiments and obtain stable impalements.

**Experimental Design**

Organ bath studies. CHARACTERIZATION OF MECHANICAL RESPONSES FOLLOWING STIMULATION OF EMNS IN THE LES AND EB. The neural responses in clasp and sling regions of LES were compared with those from EB in experiments with stimulation of EMNs by EFS or through nicotinic acetylcholine receptors (nAChRs). Transmural EFS (pulses of 0.4-ms duration, frequency 0.3–20 Hz) were applied to LES and EB preparations in 5-s trains at 26 V (7). Experiments were conducted during electrical stimulation of both excitatory and inhibitory EMN by EFS, with sequential addition of antagonists to assess the nature and effects of the neurotransmitters released. First, the nitriergic and cholinergic components of EFS responses were sequentially blocked by 1 nM L-NNA and 1 μM atropine. In the LES, the effect of 10 μM MRS2179 and 10 μM α-chymotrypsin on L-NNA-resistant EFS relaxation was assessed. In the EB, the effect of nitriergic, cholinergic, VIP, and P2Y1 antagonists was also assessed on EFS responses. The effect of single doses of 100 μM nicotine on LES strips was also assessed (7). After washing the strips with 45 ml of fresh buffer, there was a 30-min period before the following exposure. Repetitive additions of nicotine did not result in desensitization of nAChRs (7). The neurotransmitters released by stimulation of EMN through nAChRs were characterized by sequential addition of L-NNA and atropine, and the effect of MRS2179 or α-chymotrypsin on L-NNA-resistant nicotine relaxation was also assessed. Drugs were added to the baths 30 min before the stimulation of inhibitory EMN with nicotine.

**EFFECT OF AGONISTS FOR PUTATIVE EXCITATORY AND INHIBITORY NEUROTRANSMITTERS IN THE LES.** The dose-related effects on LES tone of the NO donor SNP (1 nM to 100 μM), the P2Y1 agonist ADPβS (1–100 μM), the P2X agonist α,β-meATP (1–100 μM), and VIP (1 nM to 1 μM) were assessed in the presence of the neurotoxin TTX (1 μM). Concentration-related curves of the effects of agonists were obtained by exposing LES strips to single doses of agonists for up to 3 min (reported concentrations are final bath concentrations). After washing the strips with 45 ml of fresh buffer, there was a 30 min period before the next exposure.

Electrophysiological studies. Two different protocols with different EFS parameters were performed during electrophysiological studies. The first protocol consisted of an electrical stimulus with the following characteristics: total duration, 100 ms; frequency, 20 Hz; pulse duration, 0.3 ms; and increasing amplitude voltage, 5, 10, 12, 15, 17, 20, 25, 30, and 50 V. The amplitude and the duration of EFS-induced JIP were measured under control conditions and after addition of each drug. Second, longer pulses of 5 s at supramaximal voltage of 50 V and pulse duration of 0.3 ms were performed at 1 Hz (5 pulses) and 5 Hz (25 pulses). These longer pulses elicited a longer monophasic hyperpolarization (10). The EFS-induced hyperpolarizations (JIPs)
were evaluated before and after the sequential addition of atropine (1 μM), MRS2179 (10 μM), α-chymotrypsin (10 U/ml), and L-NNA (1 mM). The effect of these drugs on the resting membrane potential (RMP) was also measured.

Data Analysis

Studies were designed to 1) study and compare pairs of clasp and sling strips from the same specimen; 2) compare LES and EB responses; and 3) explore the nitrergic and nonnitrergic inhibitory conenurotransmission in human LES and EB. The effect of EFS or pharmacological agents was determined in terms of changes in total tone. Relaxation was expressed in grams and/or in the percent change in total LES tone. Contraction was expressed in grams. The frequency of spontaneous IJP (sIJP) was measured by the standard deviation (SD-sIJP; expressed in mV) (11). The number of experiments was represented by n. Data are expressed as means ± SE. Student’s t-test was selected for comparisons, using the paired mode when appropriate. To further characterize EFS responses, the effect of pharmacological agents on frequency-response curves was performed using two-way repeated-measures ANOVA. When the t-test was significant, the Bonferroni test was carried out to determine the frequencies of statistically different responses. A P value < 0.05 was considered statistically significant. The differences between the amplitude and duration of the IJPs before and after drug infusion were compared by two-way analysis of variance (drug and voltage), followed by Bonferroni post hoc test, using GraphPad prism 4 (version 4.01, USA). A P value <0.050 was considered statistically significant.

RESULTS

Organ Bath Studies in the LES

Basal tone. Total tone after the equilibration period in clasp strips (10.05 ± 0.7 g, n = 20) was higher than tone in sling fibers (4.48 ± 0.52 g, n = 10, P < 0.05). L-NNA (1 mM) increased total tone by 35.5 ± 6.1% in the clasp and only by 18.1 ± 1.9% in the sling region (P < 0.05). Atropine (1 μM) did not significantly reduce LES tone in either clasp or sling fibers (not shown).

EFS responses. LES strips from both clasp and sling regions responded to EFS with a sharp relaxation during electrical stimulus (“on”-relaxation), followed by a phasic contraction at the end of the stimulus (“off”-contraction) (Fig. 1). The amplitude of both responses was frequency dependent (Fig. 1). Amplitude of “on-relaxation” was greater in clasp strips than in sling fibers (−91.55 ± 3.4% of total tone in clasp vs. −43.32 ± 6.3%; in sling at 5 Hz, n = 4, P < 0.05). In contrast, the amplitude of “off-contraction” was greater in sling strips than in clasp fibers (3.63 ± 1.1 g sling vs. 0.35 ± 0.3 g in clasp, P < 0.05 at 5 Hz, n = 4). In clasp strips (Fig. 1), L-NNA (1 mM) fully abolished EFS on-relaxation at all frequencies tested (n = 4, P < 0.001) and switched EFS responses to an “on”-contraction of enhanced amplitude and an “off”-relaxation of similar amplitude to on-relaxation in control experiments (Fig. 1). Subsequent addition of atropine fully abolished EFS on-contraction at all frequencies tested (n = 4, P < 0.001), and a prominent off-relaxation remained (Fig. 1). This persistent off-relaxation following L-NNA and atropine was significantly antagonized, but not completely abolished, by the P2Y1 antagonist MRS2179 at 3–10 Hz (−60.16 ± 4.2% at 5 Hz, n = 5, P < 0.05) (Fig. 2A). In independent experiments (Fig. 2B), we also found the peptidase α-chymotrypsin 10 U/ml, which cleaves VIP at the level of tyrosine residues, strongly antagonized EFS relaxation at 3–20 Hz following L-NNA and MRS2179. In sling muscle, 1 mM L-NNA switched the off-

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Fig. 1. Quantitative aspects of responses induced by electrical stimulation of enteric motoneurons (EMNs) in clasp (A) and sling (B) regions of human lower esophageal sphincter (LES). Amplitude of electrical field stimulation (EFS) “on”-relaxation was higher in clasp strips, and amplitude of EFS “off”-contraction was higher in sling strips. In both regions, N^•-nitro-l-arginine (L-NNA) changed the response to a cholinergic “on”-contraction. Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001, #P < 0.05 vs. clasp.
response from a contraction to a relaxation of lesser amplitude than the on-relaxation observed in the control experiments, and the on-relaxation to an on-contraction of greater amplitude than in control experiments. In contrast, amplitude of off-relaxation was strongly reduced by L-NNA compared with sling strips in control experiments (n = 4, P < 0.001, Fig. 1). In clasp strips, subsequent addition of atropine fully abolished EFS contraction, and no relaxation remained (Fig. 1).

Nicotine responses. Nicotine (100 μM) induced a sharp relaxation in both clasp and sling muscles (Fig. 3, A and B). The amplitude of nicotine-induced relaxation was higher in clasp strips than sling fibers (-96.73 ± 2.9 vs. -65.02 ± 1.2% of total tone, n = 4, P < 0.05). In clasp strips, following L-NNA, nicotine induced a biphasic response with a weak contraction followed by a strong relaxation (Fig. 3C). This nonnitrergic relaxation induced by stimulation of inhibitory EMNs through nAChRs in clasp fibers was significantly antagonized by MRS2179 (-58.48 ± 12.2%, n = 5, P < 0.01). In an independent group of experiments also on clasp strips, MRS2179 failed to antagonize the nicotine relaxation. However, sequential addition of L-NNA after MRS2179 caused a strong inhibition of nicotine relaxation (n = 4, -80.25 ± 7.1%), exceeding that caused by L-NNA alone (P < 0.05, Fig. 3D). Also in clasp strips, α-chymotrypsin (10 U/ml) antagonized the nonnitrergic relaxation induced by nicotine by -52.1 ± 1.9%, n = 3, and the relaxation induced by nicotine following L-NNA and MRS2179 by -72.9 ± 13.5%, n = 3 (Fig. 3E).

Effect of putative inhibitory neurotransmitters in the LES. We explored the direct effect of the proposed inhibitory neurotransmitters in clasp LES strips pretreated with the neurotoxin TTX, and we found that the NO donor, SNP (1 nM to 100 μM, n = 4), and VIP (1 nM to 1 μM, n = 3) induced a strong monophasic relaxation, the P2Y1 receptor agonist, ADPβS (1–100 μM, n = 4), induced a weak, fast, and transient relaxation, followed by a contraction, and the purinergic P2X agonist, α,β-meATP (1–100 μM, n = 3), induced a sustained contraction (Fig. 4). Strips from each region of the LES show similar concentration-dependent profiles induced by the agonist SNP, the main putative inhibitory neurotransmitter in human LES. The EC50 and the maximal effect of the relaxation of total tone induced by SNP in clasp fibers (0.41 μM, -85.4 ± 4.3%, n = 3) were of similar magnitude to those induced by SNP in sling fibers [0.59 μM, -87.9 ± 8.5%, n = 3, P = not statistically significant (NS)].

Organ Bath Studies in the EB

Strips from the EB did not develop active tone and presented much less total tone than LES strips at the end of the equilibration period (2.7 ± 0.2 g, n = 16, P < 0.05 vs. clasp and sling fibers). EB strips responded to electrical stimulation of EMNs with a brief on-contraction at the beginning of the electrical stimulus and a more prominent off-contraction at the end of it (Fig. 5). The amplitude of both contractions was frequency dependent, and off-contractions were greater than on-contractions (n = 6, P < 0.001, Fig. 5). The latency period of off-contractions was measured as the time from initiation of the electrical stimulus to the onset of the contraction, and this latency increased with the frequency of electrical stimulation (Fig. 5). Blockade of P2Y1 receptors by MRS2179 (10 μM)
decreased amplitude of both on- \((20 \text{ Hz, } -55.42 \pm 8.1\%, P < 0.05)\) and off-contractions \((20 \text{ Hz, } -39.37 \pm 10.1\%, P < 0.001)\), without affecting latency at all frequencies except 0.3 Hz, \(P < 0.05\) (Fig. 5). In addition, \(\alpha\)-chymotrypsin similarly reduced the amplitude of the on- \((20 \text{ Hz, } -50.5 \pm 10.0\%, P < 0.05)\) and off-contractions \((20 \text{ Hz, } -35.7 \pm 6.4\%, P < 0.001)\) without affecting latency at all frequencies (not shown). In contrast, L-NNA \((1 \text{ mM})\) completely abolished the latency of off-contractions, inducing the appearance of on-contractions of enhanced amplitude during the electrical stimulus (Fig. 5). We compared the amplitude of the esophageal contractions induced by electrical stimulation at 10 and 20 Hz in the EB with

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Fig. 3. Responses to stimulation of EMNs through nicotinic acetylcholine receptors \(\text{(nAChRs)}\) in human clasp and sling LES regions. Nicotine \((\text{NIC})\) induced a strong relaxation in both clasp \((n = 4; A)\) and sling \((n = 4; B)\) LES sides. \#\(P < 0.05\), sling control vs. clasp control. \(A\): following L-NNA, NIC still induced an intense relaxation in clasp region \((n = 4)\). Nonsignificant, and \(*\*\*P < 0.001\) vs. control contraction. \(B\): in contrast, L-NNA fully abolished NIC relaxation in sling strips \((n = 4)\). \(*P < 0.05\) vs. control relaxation or control contraction. \(C\): in clasp strips, 10 \(\mu\text{M} MRS2179\) antagonized NIC relaxation following L-NNA, \(*\*\*P < 0.01\) vs. nonnitrergic relaxation. \(D\): the antagonism of NIC relaxation by MRS2179 and L-NNA exceeded that of L-NNA alone \((n = 5)\). \(E\): 10 U/ml \(\alpha\)-CT antagonized the relaxation induced by NIC following L-NNA and MRS2179 \((n = 3)\). \(*\*\*P < 0.001\) vs. control relaxation. \(*\*P < 0.01\) vs. control contraction. \(*P < 0.05\) vs. L-NNA + MRS2179 relaxation or contraction. Values are means \(\pm\ SE.\)
those induced by the same electrical stimuli in clasp and sling fibers in the LES (Fig. 6). Amplitude of electrical-induced off-contractions, as well as amplitude of electrical on-contractions following \( \text{1-NNA} (1 \text{ mM}) \) was higher in the EB than in both clasp and sling sides of the LES (Fig. 6). As on-contraction following \( \text{1-NNA} \) is almost completely blocked by atropine in the EB and clasp and sling fibers in the LES, these results suggest that the effect of cholinergic neurons was stronger in the EB than in either LES side.

**Electrophysiological Studies in the LES and EB**

The nature of inhibitory neurotransmitters in clasp LES and EB fibers was also explored in electrophysiological studies. RMP in clasp \((-43.9 \pm 0.4 \text{ mV, } n = 4) \) and sling \((-42.66 \pm 1.91 \text{ mV, } n = 3) \) regions of the LES was similar, and both were significantly less negative than the RMP in the EB \((-52.5 \pm 1.7 \text{ mV, } n = 4, P < 0.01 \text{ vs. clasp and sling}) \). The RMP in the clasp LES and EB presented continuous small oscillations (unitary potentials, Figs. 7 and 8) and low SD-sIJP values of \( 0.079 \pm 0.047 \text{ mV (} n = 4) \) in the clasp, \( 0.16 \pm 0.026 \text{ mV (} n = 3) \) in the sling, and \( 0.13 \pm 0.05 \text{ mV (} n = 5) \) in the EB (NS), suggesting that there are very few sIJPs in the human esophagus under these experimental conditions. Neither atropine, MRS2179, nor \( \text{1-NNA} \) affected RMP or SD-sIJP values in the EB. The \( \text{1-NNA} \) was ineffective, showing that NO mediates the transient hyperpolarization in the human LES and EB (Figs. 7 and 8).

DISCUSSION

In this study, we found that human LES relaxation induced by stimulation of inhibitory motoneurons of the myenteric plexus is mainly mediated by neural NO with a minor contribution of a purine acting through \( \text{P2Y}_1 \) receptors and VIP. In contrast, the IJP in the LES and EB, and latency of EB contractions, are fully mediated by NO, and we could not find a purinergic or VIP-ergic component in the IJP. We also found that the human esophagus presents a significant intrinsic regional specialization at the myenteric plexus level. These results in the human LES agree with our laboratory’s previous studies on pigs (27). EFS caused strong stimulation of inhibitory EMNs in the clasp region, as relaxation was greater than in the sling region (15). In contrast, EFS in the sling region induced a less efficient relaxing response and a more intense contraction. In the clasp region, nicotine caused strong stimulation of nitricergic EMNs and induced a relaxation similar to that obtained by maximal EFS. In the sling region, stimulation of inhibitory EMNs through \( \text{nACHRs} \) caused a higher relaxation than that obtained by maximal EFS; however, the amplitude of this relaxation was lower than that obtained in clasp strips. In contrast, the direct effect of inhibitory NTs is similar between both LES sides, suggesting this functional asymmetry can be attributed to specialization at the myenteric plexus level. These results in the human LES agree with our laboratory’s previous studies on pigs (8) and previous studies in cats (16), which found that stimulation of EMNs induces stronger inhibitory responses in clasp fibers and stronger excitatory responses in sling fibers. Supporting our results on both humans and pigs, studies on the guinea pig LES also show a marked functional neural asymmetry, as vagal stimulation causes activation of excitatory and inhibitory EMNs on the sling LES side (40) and only inhibitory responses in the clasp (39). In addition, all of these functional studies and our present results on humans agree with morphological studies mapping the regional distribution of EMNs in guinea pig LES that found that, in the clasp region, 33% of the

![Diagram](https://via.placeholder.com/150)
neurons stain positively for cholineacetyltransferase, whereas 70% stain positively for NO synthase (2). In contrast, sling fibers are mainly innervated (>80%) by cholinergic neurons, with only 15% staining positively for NO synthase (40, 2).

Taken together with our previous functional studies, all of these findings strongly suggest that, in the human LES, the inhibitory neurons predominate in the clasp, whereas the excitatory ones predominate in the sling fibers.

LES responses to EFS and nicotine in this study are similar to those our laboratory found in earlier studies on the human LES (12). Then we found NO was the main mediator for LES relaxation in the human LES and that other minor apamin-sensitive inhibitory con neurotransmitters were released (12). In the present study, both the EFS on-relaxation observed in organ bath studies and the IJP observed in electrophysiological studies were fully abolished by NO synthesis inhibitors, further confirming a major role for NO in LES hyperpolarization and subsequent relaxation of human LES following stimulation of inhibitory EMNs. We also conducted additional experiments showing that adding L-NNA fully abolished latency of off-relaxations, inducing the appearance of on-relaxations of enhanced amplitude (n = 6). Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 vs. each control. ##P < 0.01, ###P < 0.001, off-contraction vs. on-contraction.

Fig. 5. Representative tracings (A) and frequency-related responses on amplitude (B) and latency (C) of esophageal body (EB) contractions. Strips from the EB responded to electrical stimulation of EMNs with an on-contraction at the beginning of the stimulus and an off-contraction following the end of the stimulus. Blockade of P2Y1 receptors by MRS2179 decreased amplitude of both on- and off-contractions without affecting latency. In contrast, L-NNA fully abolished latency of off-relaxations, inducing the appearance of on-relaxations of enhanced amplitude (n = 6). Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 vs. each control. ##P < 0.01, ###P < 0.001, off-contraction vs. on-contraction.
sustently inhibited by the P2X receptor antagonist NF279. We have now found, in the human LES, that the P2Y1 agonist ADPβS caused a relaxation of LES, whereas the P2X agonist caused a contraction. In addition, we found the nonnitric off-relaxation following stimulation of EMNs by EFS or through nAChRs was significantly inhibited by the P2Y1 antagonist MRS2179. In our study, VIP also caused a strong contraction. In addition, we found the nonnitrergic and nonpurinergic relaxation following stimulation of inhibitory motoneurons by EFS or through nAChRs was antagonized by nonpurinergic relaxation following stimulation of inhibitory myenteric neurons controlled both the amplitude and latency of the off-response in the distal smooth muscle esophagus behaving, in vitro, as a sphincter. Further studies on humans combining morphological (electron microscopy, immunohistochemistry) and functional methods (in vivo and in vitro physiology) will clarify this point in the next few years. Although our study has not been specifically designed to solve this controversy, we have not found circular fibers at the end of the human esophagus (lower esophageal circular) (19, 35). There are those at one extreme of the controversy who do not believe that the clasp-sling muscle fiber complex (7, 12, 15–17, 27, 31, 32). Another group of researchers considers the LES to be the clasp and sling muscle fiber complex (14, 41). A similar IJP was also described in the opossum, where the fast component presents anatomic similarities to the human LES (42). In our study, electrical stimulation of EMNs produced a low-amplitude monophasic IJP in both clasp and sling strips, fully abolished by t- NNA and similar to that observed in the EB. The IJP in human LES and EB has different properties than the IJP we recorded in response to identical electrical stimuli from other areas of the human gastrointestinal tract. In the colon (9, 10) and small intestine (unpublished observations), the IJP has a fast component of ~20–40 mV, which is sensitive to P2Y1 antagonist, followed by a slow component of ~10–20 mV, which is mainly nitric. The fast component presents a rundown, whereas the slow component can be sustained (10). In the clasp region of the mouse (14, 41), IJP also has a fast component partly antagonized by apamin, followed by a slow sustained nitric component. In the present study, we could not record a biphasic IJP, and the response in strips obtained from both the EB and the clasp and sling regions in the LES showed only a slow component with a small amplitude (5–6 mV), which was t- NNA sensitive and could be sustained under repetitive stimulation. A similar IJP of small amplitude has also been described in the opossum, which presents anatomic similarities to the human LES (42). In our study, we could not find a purinergic or VIP-ergic component in the IJP responsible for nonnitric relaxation in clasp fibers. This can be attributed to the different electrical stimuli we used in organ bath or microelectrode experiments to stimulate EMNs or to a putative nonelectromechanical coupling in the t- NNA-resistant inhibitory pathway. It is important to notice that the RMP of clasp (~44 mV) and sling muscle (~42 mV) was more positive than that of the EB (~53 mV). This difference might be responsible for the myogenic tone present in sphincters, which is absent in the EB. Taken together, our results suggest that on-relaxation and the IJP in the human LES is caused by NO release from inhibitory EMNs, and there is a minor nonnitric off-relaxation mainly mediated by the release of both a purine, also from inhibitory EMNs acting at muscular P2Y1 receptors, and VIP.

There is some controversy in the literature regarding the definition of the LES from both an anatomic and physiological perspective, and it seems that researchers in this field have developed two different viewpoints. One group of researchers consider the LES to be the clasp and sling muscle fiber complex (7, 12, 15–17, 27, 31, 32). Another group of researchers consider the LES to be the circular smooth muscle near the distal end of the esophagus (lower esophageal circular) (19, 35). There are those at one extreme of the controversy who do not believe that the clasp-sling muscle fiber complex pertains to the LES (35), and at the other extreme who do not believe in the lower esophageal circular even exists. We believe part of the discrepancy is due to differences between humans and the animal species used in some of the previous studies, and another important part of this controversy is due to different methodological approaches (in vivo vs. in vitro). Although our study has not been specifically designed to solve this controversy, we have not found circular fibers at the end of the human esophagus behaving, in vitro, as a sphincter. Further studies on humans combining morphological (electronic microscopy, immunohistochemistry) and functional methods (in vivo and in vitro physiology) will clarify this point in the next few years. Circular esophageal muscle fibers from the EB respond with a minor contraction during the electrical stimulus, “on-contraction”, and a prominent “off-contraction” shortly after the end of the stimulus. The time between the beginning of the stimulus and the initiation of the on- and off-contraction is termed latency (1). Animal studies described that NO released from inhibitory myenteric neurons controlled both the amplitude and latency timing of the off-response in the distal smooth muscle esophagus (5) and mediated nerve-induced hyperpolarization of circular esophageal and LES smooth muscle (4, 6).
Fig. 7. Intracellular microelectrode recordings [EFS-induced inhibitory junction potential (IJP) elicited at 50 V] and voltage-dependent curves showing the sequential effect of 1 μM atropine, 10 μM MRS2179, and 1 mM L-NNA on the amplitude and duration of the EFS-induced IJP in clasp (A) and sling (B) LES strips. Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.
These in vitro animal studies also found that inhibition of NOS with L-NNA or by blocking the NO-intracellular pathway with the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinolin-1-one attenuated or even abolished the off-response and shortened the latency period, inducing the appearance of a cholinergic on-contraction of greater amplitude than the previous on- and off-contractions (5, 20). In our human study, in vitro on and off EB contractions are similar to these previous in vitro animal studies and to the two types of esophageal peristaltic contractions found during in vivo vagal stimulation in the opossum esophagus (20). The contraction during vagal stimulation was primarily cholinergic and was called the “A wave.” The other contraction occurred after stimulus and was called the “B wave.” NOS inhibitors strongly increased the velocity of the cholinergic A wave by decreasing the time of its arrival in the distal esophagus (38), a very similar result to what we found on latency in the human EB. Animal studies also found that the amplitude of esophageal peristalsis is controlled by a peripherally located gradient of cholinergic innervation, most prominent proximally, and which decreases distally along the smooth muscle portion of the esophagus (5). Our results also suggest a complex interplay between the excitatory cholinergic and the inhibitory nitrergic systems in the control of esophageal peristalsis in the human esophagus. Our electrophysiological results in the human esophagus show that the IJP in the EB is fully nitrergic, as well as latency of off-contractions, suggesting that nitrergic EMNs control the velocity of peristalsis. We also found that the RMP of the human EB muscle is more “negative” compared with that of clasp LES fibers, agreeing with the differences observed in the tone of both regions (39, 41). Blockade of NO synthesis in the EB strips shifted off-contraction to an enhanced cholinergic on-contraction, suggesting an inhibitory effect of nitrergic EMNs on amplitude of EB contractions, as well as a predominant role of cholinergic neurons in the amplitude of EB peristalsis. We found decreasing effects of the intrinsic cholinergic innervation from the EB to both sides of human LES, supporting the concept that amplitude of esophageal contractions is determined by a balance of these intrinsic circuits.

Finally, the P2Y1 receptor antagonist MRS2179 did not affect latency, but reduced amplitude of off-contractions, suggesting a modulation of cholinergic neurons by excitatory P2Y1 receptors (1).

Alterations in these intrinsic mechanisms controlling LES and EB physiology contribute to the pathophysiology of gastroesophageal reflux disease and achalasia. Recent studies found that the smooth muscle pressure component of the LES caused by the clasp and sling muscle fibers is defective in

Fig. 8. A: intracellular microelectrode recordings showing the sequential effect of 1 μM atropine, 10 μM MRS2179, and 1 mM L-NNA on the EFS-induced IJP elicited at the maximum voltage of stimulation (50 V) in the EB. B: plot graphs show the amplitude and duration of the IJP at 5–50 V of stimulation. Values are means ± SE. *P < 0.05, ***P < 0.001.
Fig. 9. Intracellular microelectrode recordings in clasp (A) and sling (B) LES strips and EB (C) showing the EFS-induced IJPs obtained during 5-s stimulation at 5 Hz (25 pulses) and 1 Hz (5 pulses). Atropine (1 μM) and MRS2179 (10 μM) did not affect either amplitude or duration of these IJP, whereas L-NNA (1 mM) fully abolished the IJP in clasp, sling, and EB regions.
patients with gastroesophageal reflux disease (19). Cytokines and free radicals might mediate esophagitis-associated impairment of cholinergic EMNs, decreasing LES tone and amplitude of esophageal peristalsis (31). Otherwise, the imbalance between a preserved excitatory and an impaired intrinsic inhibitory innervation (25) might explain why patients with achalasia show a hypertensive and nonrelaxing LES, greatly increased tone in the LES, and nonprogressive simultaneous contractions in the EB (18,32).

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

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