Pharmacologic Specificity of Nicotinic Receptor-Mediated Relaxation of Muscarinic Receptor Precontracted Human Gastric Clasp and Sling Muscle Fibers within the Gastroesophageal Junction

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ABSTRACT

Relaxation of gastric clasp and sling muscle fibers is involved in the transient lower esophageal sphincter relaxations underlying the pathophysiology of gastroesophageal reflux disease (GERD). These fibers do not contribute tone to the high-pressure zone in GERD patients, indicating their role in pathophysiology. This study identifies some mediators of the nicotine-induced relaxation of muscarinic receptor precontracted gastric clasp and sling fibers. Muscle strips from organ donors precontracted with bethanechol were relaxed with nicotine and then rechallenged after washing and adding inhibitors tetrodotoxin (TTX), the nitric-oxide synthase inhibitor L-nitro-arginine methyl ester (l-NAME), the β-adrenoceptor antagonist propranolol, the glycine receptor antagonist strychnine or ginkgolide B, and the GABA<sub>A</sub> receptor antagonist bicuculline or 2-(3-carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide [(gabazine) SR95531]. TTX only inhibited clasp fiber relaxations. l-NAME and propranolol inhibited, and ginkgolide B was ineffective in both. SR95531 was ineffective in clasp fibers and partially effective in sling fibers. Strychnine and bicuculline prevented relaxations with low potency, indicating actions not on glycine or GABA<sub>A</sub> receptors but more consistent with nicotinic receptor blockade. Bethanechol-precontracted fibers were relaxed by the nitric oxide donor S-nitroso-N-acetyl-L-penicillamine and by the β-adrenergic agonist isoproterenol (clasp fibers only) but not by the glycine receptor agonist taurine or glycine or the GABA<sub>A</sub> agonist muscimol. These data indicate that nicotinic receptor activation mediates relaxation via release of nitric oxide in clasp and sling fibers, norepinephrine acting on β-adrenoceptors in clasp fibers, and GABA acting on GABA<sub>A</sub> receptors in sling fibers. Agents that selectively prevent these relaxations may be useful in the treatment of GERD.

Introduction

A complex anatomy, physiology, and mechanics mediate functions of the gastroesophageal sphincter that are necessary to regulate esophageal emptying into the stomach and to permit air venting while protecting against unwanted reflux of gastric content. The relative tonic contributions to the protective function of the gastroesophageal sphincter and the relative loss of tone during transient lower esophageal sphincteric relaxations (TLESR) underlie sphincteric function (Dent et al., 1980; Boeckxstaens, 2005).

The circular muscle fibers at the distal end of the esophagus have been traditionally considered to be the lower esophageal sphincter (Kahrilas, 1997; Cheng et al., 2005; Mittal...
and Goyal, 2006). However, the first barrier to gastric reflux is actually the clasp and sling muscle fiber complex of the proximal stomach. Several investigators also use this same term “lower esophageal sphincter” (LES) to indicate this clasp and sling muscle fiber complex (Gonzalez et al., 2004; Tian et al., 2004). The intrinsic inner circular muscles of the distal 2 to 3 cm of the esophagus (the lower esophageal circular muscles, LEC) together with the gastric sling/clasp fiber muscle complex constitute the internal, smooth muscle mechanism of the high-pressure zone. The crural diaphragm, positioned between and overlapping these two anatomically distinct smooth muscle structures, constitutes the external skeletal muscle mechanism. The sling/clasp muscle complex has been described as a thickened wall of smooth muscle at the gastroesophageal junction (GEJ) in cadaver studies (Lieberman-Meffert et al., 1979; Stein et al., 1991, 1995). Sling muscle fibers surround the junction of the esophagus on the greater curvature side of the gastric cardia. Clasp muscle fibers, found on the lesser curvature junction of the stomach, attach to the sling muscle. It has been proposed that the perfect match between the manometric pressure and the gastric sling/clasp complex indicates that the gastric sling/clasp muscle fiber group is actually the anatomic correlate of the manometric lower esophageal sphincter (Stein et al., 1995). However, because these muscles are anatomically part of the stomach and not the esophagus, a more appropriate name for the clasp/clasp smooth muscle fiber complex is the “upper gastric sphincter.” Because the lower esophageal circular smooth muscle component, the crural diaphragm, and the gastric sling/clasp muscle fiber complex are anatomically contiguous in normal individuals, intraluminal pressure is attributed to contractions of a combination of all of these muscles.

Many of the published studies on relaxation of gastrointestinal smooth muscle focused on either electric field stimulation (EFS)-induced relaxations or inhibition of stretch-induced active tone. It has been known for at least 50 years that the pressure of the human gastroesophageal high-pressure zone (HPZ) is reduced by administration of the muscarinic receptor antagonist atropine (Betarettolo et al., 1960; Skinner and Camp, 1968; Opie et al., 1987). The pressure generated specifically by the human clasp and sling muscle fiber complex as well as the lower esophageal circular muscle in vivo is reduced by atropine (Miller et al., 2009). These findings suggest that the in vivo tone of the HPZ is maintained by the neuronal release of acetylcholine acting on smooth muscle muscarinic receptors. In vitro stretch-induced tone in muscle strips from human GEJ is active and calcium-sensitive (Kovac et al., 2005). However, this stretch-induced tone is not decreased by atropine (Preiksaitis and Diamant, 1997; Gonzalez et al., 2004). Therefore, in vitro stretch-induced tone develops with a different mechanism than the in vivo atropine-sensitive tone that is responsible for generating in vivo pressure in the gastroesophageal HPZ. Thus, relaxation of muscarinic receptor-precontracted clasp and sling muscle may be the in vitro physiologic equivalent of in vivo TLESR and deglutitive inhibition.

A number of investigators have demonstrated that nicotine induces relaxation of stretch-induced tone. To the best of our knowledge, no similar studies have been performed on bethanechol-precontracted clasp and sling muscle strips. Nicotine causes relaxation of stretch-induced tone indirectly through various secondary mediators and signal transduction pathways. For these reasons, we chose to study nicotine-induced relaxations of bethanechol-precontracted clasp and sling muscle fibers. This paradigm allows use of specific agonists and antagonists to explore the secondary messengers that mediate nicotine-induced relaxation of clasp and sling muscle fibers. These fibers relax during transient lower esophageal sphincter relaxations that underlie the pathophysiology of gastroesophageal reflux disease (GERD).

Materials and Methods

All drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO). A total of 14 specimens of human stomach with attached esophagus were obtained from subjects with no known history of GERD. The mean age of the subjects was 46.6 ± 5.9 years, 11 of which were men and 3 were women, 9 of the subjects were Caucasian, 2 were Hispanic, 1 was African American, 1 was Asian, and 1 was Indian. The average height and weight were 174 cm and 86.4 kg, respectively. We obtained these specimens from brain-dead patients maintained on life support who consented to organ transplant donation. Their next of kin consented to the donation of nontransplantable organs for research. These human organ specimens were obtained from organ procurement agencies (National Disease Research Institute and the International Institute for the Advancement of Medicine). The specimens were harvested within 30 min after cross-clamping the aorta. The stomach contents were gently rinsed out with saline. The esophageal and pyloric openings were ligated, and the entire specimen was transported to our laboratory on ice by overnight courier immersed in either University of Wisconsin (Beltzer’s Viaspan) organ transport media (University of Wisconsin) or HTK, which was composed of 15 mM NaCl, 9 mM KCl, 1 mM potassium hydrogen 2-ketoglutarate, 4 mM MgCl2, 18 mM histidine NaCl, 2 mM tryptophan, 30 mM mannitol, and 0.015 mM CaCl2·2H2O.

Preparation of Smooth Muscle Strips. The specimens were dissected in a cold room (0–5°C). The greater and lesser omentum were removed. The outermost longitudinal fibers descending from the esophagus across the stomach were removed by sharp dissection. This exposed the sling muscle fibers as a U-shaped group of fibers approximately 8 mm wide enveloping the esophagus around the greater curvature of the stomach and the semicircular clasp muscle fibers around the lesser curvature opposite to the cardiac notch. The clasp muscle fibers are oriented perpendicular to the sling muscle fibers and connect between the open ends of the U-shaped sling muscle fibers. Beginning at the cardiac notch, the sling muscle fiber complex was separated from the underlying submucosa by sharp dissection, and this tissue plane was followed completely around the lesser curvature, thus separating the clasp muscle fibers from underlying submucosa. The clasp muscle fibers were removed from the smin fibers by sharp dissection and cut into 18 to 24 smooth muscle strips approximately 2 × 2 × 8 mm with the long axis parallel to the direction of the muscle fibers. Similar muscle strips (32–48) were cut from the sling muscle fibers. The LEC fibers (32–48) were obtained from the thickened area of the esophagus approximately 1 to 2 cm proximal to the stomach. At the conclusion of the experiment, each muscle strip length between the suspending clips was measured with a caliper and cut off from the suspending clips, and the tissue participating in the contractile response was weighed. The average length and weight of the muscle strips were 7.9 ± 0.2 mm and 7.6 ± 0.3 mg for the clasp fibers; 8.9 ± 0.2 mm and 8.8 ± 0.4 mg for the sling fibers, and 8.0 ± 0.1 mm and 13.7 ± 0.5 mg for the LEC fibers. These dissections were performed to isolate the clasp, sling, and LEC fibers and avoid the confounding effects of the overlying longitudinal smooth muscle or underlying mucosal or submucosal layers.

Contraction and Relaxation Responses of Smooth Muscle Strips. Responses of the clasp muscle fibers were studied first fol-
lowed by sling fibers and then LEC fibers. The specimen remained on ice immersed in the organ transport medium in the interim. These smooth muscle strips were suspended in 10-ml muscle baths in Tyrode’s solution continuously bubbled with 95% O2/5% CO2 and maintained at 37°C. The length of the muscle strips were adjusted over 15 min to produce 1 g of basal tension, and then strips were allowed to accommodate to the muscle bath for an additional 15 min. The strips were then washed by three exchanges of fresh bathing solution and allowed an additional 15 min of accommodation before investigation of their contractile response. All studies were completed within 36 h after receiving the tissues.

Strips were exposed to a near maximally effective concentration of bethanechol (30 μM) and then, after reaching a plateau in tension, nicotine was added. After a 60-min repeated washing (three bath exchanges every 15 min) and re-equilibration period, strips were exposed to potential inhibitors of relaxations for 30 min and then rechallenged with bethanechol followed by nicotine. The following compounds were tested for their ability to inhibit relaxation: the nitric-oxide synthase (NOS) inhibitor L-NAME (10, 30, and 100 μM), the β-adrenergic receptor antagonist propranolol (1, 5, and 10 μM), the glycine receptor antagonist strychnine (3, 10, and 30 μM) and ginkgolide B (10 μM), the GABA<sub>A</sub> receptor agonists bicuculline (methylisodide salt, 10, 30, and 100 μM) and SR95531 (30 μM), and the sodium channel blocker tetrodotoxin (1 μM). Agonists of these receptor systems were also added to separate strips after inducing a plateau of tension with 30 μM bethanechol. This included the nitric oxide donor S-nitroso-N-acetyl-DL-phenylhydrazine (SNAP) (1, 10, and 100 μM), the β-adrenoceptor agonist isoproterenol (1, 10, and 100 μM), the GABA<sub>A</sub> receptor agonist muscimol (10, 100, and 1000 μM), and the glycine agonists taurine and glycine (0.1, 1, and 10 mM).

**Immunohistochemistry.** After the above experiments were performed, muscle strips were flash-frozen on dry ice, cryosectioned into 12-μm longitudinal sections, mounted onto charged slides (Fisher Plus slides), and then fixed for 30 min in 4% paraformaldehyde in PO<sub>4</sub> buffer, pH 7.4. After drying overnight, adjacent sections on slides were washed in phosphate-buffered saline (PBS), incubated in 3% H<sub>2</sub>O<sub>2</sub> in methanol (4°C) for 30 min, washed, incubated in 10% goat serum with 4% dried milk in PBS and 0.3% Triton X-100 for 30 min, and then incubated overnight at 24°C with either a NeuN antibody (a neuronal nuclear marker [Millipore, Billerica, MA]; 1:200 dilution in PBS) or anti-PGP9.5 (a pan-neuronal marker [Abcam, Cambridge, MA]; 1:50 dilution in 10% goat serum in PBS). After washing, sections were incubated for 2 h at 24°C with goat anti-mouse peroxidase-conjugated (HRP) secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 with PBS. HRP was visualized as a black immunoreactive stain using diaminobenzidine with cobalt (Sigma-Aldrich). These sections were counter-stained lightly with eosin. One centimeter by 1-cm blocks of tissue (full thickness) were collected from an esophagus and stomach specimen that was fixed for 48 h in 4% paraformaldehyde in PO<sub>4</sub> buffer as a whole organ from an additional donor with no known history of GERD, equilibrated in 30% sucrose in PO<sub>4</sub> buffer for 3 days, cryosectioned into full thickness of 12-μm slices, mounted on charged slides, and stained with hematoxylin and eosin.

**Data and Statistical Analysis.** IC<sub>50</sub> values for the antagonists and EC<sub>50</sub> for agonists were determined by linear regression of the dose-effect data (Tallarida, 2000). For each condition, a minimum of 6 separate muscle strips obtained from two to four different donors were used. Statistical analysis of the contractility data were performed using analysis of variance (ANOVA) followed by Newman-Keuls post hoc tests. In the event that homogeneity of variance tests (Hartley’s F-max, Cochran’s C or Bartlett’s χ<sup>2</sup>) showed statistically significant differences in the variances between groups, the data were converted to ranks before performing the ANOVA. Differences were considered statistically significant at p < 0.05.

**Results**

Figure 1, A and B, shows hematoxylin and eosin-stained sections from the esophagus and stomach specimen that was fixed as a whole organ. Figure 1A is a cross-section of the esophagus at approximately 2 cm proximal to the gastro-esophageal junction. Figure 1B is a cross-section of the stomach at approximately 1 cm distal to the GEJ on the lesser curvature. LEC, clasp, and sling regions are indicated in the inner circular muscle layer. After the in vitro contractility assays, smooth muscle strips from these same three regions were fixed, sectioned, and probed immunohistochemically for neuronal cell bodies (NeuN+) and axons (Pgp9.5+). As shown in Fig. 1, C, D, F, G, I, and J, small NeuN+ neuronal neurons are located between individual smooth muscle fibers located within the inner circular layer of each region. Each set of neuronal cell bodies were associated with Pgp9.5+ neuronal axons (Fig. 1, E, H, and K).

All muscle strips developed decreased tone below the 1 g of basal tone during the second 15-min accommodation period (0.71 ± 0.03 for clasp, 0.51 ± 0.02 g for sling, and 0.88 ± 0.03 g for LEC fibers). At the end of the 15-min period after the wash, immediately before bethanechol addition, the basal tone was 0.54 ± 0.02 g for clasp, 0.52 ± 0.02 g for sling, and 0.69 ± 0.03 g for LEC fibers. This basal tension after the second 15-min period and the tension immediately before the first exposure to bethanechol were statistically significantly higher in LEC fibers than clasp or sling fibers (p < 0.01). The effect of exposure to nicotine was determined without pre-contracting the strips to bethanechol in a separate group of eight muscle strips from each of the three different muscle fibers. Nicotine (1 mM) caused relaxation of clasp fibers (−0.22 ± 0.07 g), contraction of sling fibers (2.02 ± 0.06 g), and relaxation of LEC fibers (−0.17 ± 0.06 g). The response of the clasp fibers was statistically significantly greater than the clasp and LEC responses (p < 0.01).

**Nicotine-Induced Relaxations of Bethanechol Pre-contracted Muscle Strips.** Bethanechol induces contraction of clasp and sling fibers with similar potency: EC<sub>50</sub> = 8.4 ± 1.8 μM for clasp, 11 ± 1.3 μM for sling, and 7.1 ± 1.2 μM for LEC fibers (Fig. 2). Based on these findings, 30 μM bethanechol was used to precontract clasp, sling, and LEC fibers to near maximal tension. Cumulative addition of nicotine (10 μM–1 mM) induces relaxation of these precontracted muscle strips (Fig. 3, open symbols). In clasp fibers, the maximal relaxation occurred at 100 μM nicotine, and in LEC fibers, maximal relaxation occurred at 30 μM. Higher concentrations induced relaxations; however, these were not as great as the effect of 100 μM, possibly because of receptor desensitization. In sling fibers, the maximal relaxation occurred at 1 mM nicotine. In separate strips from different donors, a single dose of 1 mM nicotine induced relaxations that were not statistically significantly different from the maximal relaxation obtained during the cumulative addition of nicotine (Fig. 3, closed symbols). Based on these results, a single concentration of 1 mM nicotine was used for determination of IC<sub>50</sub> values for the antagonists. Representative traces from these experiments in clasp muscle fibers are shown in Fig. 4. As can be seen in the traces at the left in Fig. 4, a relaxation is induced in response to 1 mM nicotine added after the clasp strips contracted to 30 μM bethanechol. The traces on the right in Fig. 4 show representative traces of the
blockade of these nicotine induced relaxations by the different antagonists as detailed below. Similar representative traces for sling and LEC fibers can be found in Supplemental Figs. 1 and 2, respectively.

**Inhibition of Nicotine-Induced Relaxations.** Our initial approach was to determine which inhibitors either blocked the maximal nicotine induced relaxation or shifted the nicotine concentration effect curve to the right. Thus, muscle strips were precontracted with 30 μM bethanechol and induced to relax with three cumulative additions of increasing concentrations of nicotine (0.1, 0.3, and 1.0 mM nicotine). After extensive washing and addition of either vehicle or antagonist for 30 min, the muscle strips were stimulated to contract with 30 μM bethanechol and then
relaxed to the three concentrations of nicotine. Five of the inhibitors tested: bicuculline (100 μM), L-NAME (100 μM), propranolol (10 μM), strychnine (300 μM), and tetrodotoxin ([TTX] 1 μM), significantly attenuated the maximal nicotine-induced relaxation in clasp fibers. Similar findings were found in sling and LEC fibers, with the exception that TTX did not attenuate the relaxation in sling or LEC fibers. The mean ± S.E.M. of the effect of these inhibitors on the contraction induced by bethanechol and on maximal relaxation response induced by nicotine for both clasp, sling, and LEC fibers is shown in the bar graphs of Fig. 5. Detailed concentration-response curves for nicotine with each antagonist are shown in Supplemental Figs. 3 to 5 for clasp, sling, and LEC fibers, respectively. None of the antagonists significantly affected the bethanechol-induced contraction in clasp, sling, or LEC fibers. In a separate group of strips, a single concentration (100 μM) of the ganglionic nicotinic receptor antagonist hexamethonium was tested in the three different tissues for its ability to block 1 mM nicotine-induced relaxation of 30 μM bethanechol-precontracted strips. Hexamethonium had no effect on bethanechol induced contractions but caused a statistically significant inhibition of the nicotine-induced relaxation (p < 0.05 for clasp, p < 0.01 for sling and LEC fibers; data not shown).

In a second series of experiments performed on different specimens from the initial studies described above, various concentrations of the antagonists (L-NAME, propranolol, bicuculline, and strychnine) were used to determine IC50 values. In these experiments, a single maximally effective concentration of 1 mM nicotine was used to induce relaxations of the muscle strips precontracted with 30 μM bethanechol. Shown in Fig. 6 is the effect of the individual concentrations of propranolol on both the contractile response to bethanechol and the relaxation response to nicotine for clasp, sling, and LEC fibers. Similar data were obtained for bicuculline, L-NAME, and strychnine and can be found in Supplemental Figs. 6 to 8 for clasp, sling, and LEC fibers, respectively. The relaxations were normalized to the pre-antagonist relaxations from the same muscle strip to determine the average percentage effect for each dose of antagonist (Fig. 7). The dose-effect data for each were analyzed by linear regression of effect on log dose from which the IC50 was determined as described by Tallarida (2000) and shown in Table 1. The IC50 values for L-NAME, bicuculline, and strychnine were not statistically significantly different between clasp, sling, and LEC fibers. The IC50 value for propranolol was significantly lower in sling and LEC than in clasp fibers, demonstrating that propranolol is more potent in inhibiting nicotine-induced relaxations in sling and LEC fibers than in clasp fibers.

The potencies of bicuculline and strychnine in clasp, sling, and LEC fibers (Table 1) were much lower than their reported potencies at GABA<sub>A</sub> (Maggi et al., 1984; Huang and Johnston, 1990) and glycine receptors (Lynch, 2004), respectively, and within their reported potency for antagonism of nicotinic receptors (Zhang and Feltz, 1991; Kuijpers et al., 1994; Albuquerque et al., 1998; Demuro et al., 2001). Therefore, other antagonists with no reported activity at nicotinic receptors were used: the GABA<sub>A</sub> antagonist SR95531 (Tonini et al., 1989; Zhang and Feltz, 1991) and the glycine receptor antagonist ginkgolide B (Kondratskaya et al., 2002, 2004) in six to eight separate clasp and sling muscle strips from two different donors. Ginkgolide B (10 μM) had no effect on nicotine-induced relaxations in clasp or sling fibers, and SR95531 (30 μM) was also ineffective in clasp fibers but reduced nicotine-induced relaxations by 35 ± 8% (n = 11 strips from two donors) in sling fibers.

**Agonist-Induced Relaxations of Bethanechol-Precontracted Muscle Strips.** To determine whether agonists...
of these receptor systems could mimic the nicotine-induced relaxations, strips were contracted with 30 \( \mu \)M bethanechol and then exposed to increasing concentrations of isoproterenol, SNAP, muscimol, taurine, and glycine. Neither taurine nor glycine had any relaxatory effect on plateau tension at concentrations up to 10 mM (data not shown). Figure 8 shows concentration-effect curves for muscimol, isoproterenol, and SNAP along with nicotine for comparison and time control strips that indicate the slow reduction in tension over the same time period in 30 \( \mu \)M bethanechol-contracted strips that were not exposed to any additional agents. Muscimol in both clasp and sling fibers and isoproterenol in sling fibers did not induce a greater degree of relaxation than in the time control strips and, therefore, did not mimic the nicotine-induced relaxations. SNAP in both clasp and sling fibers and isoproterenol in clasp fibers induced relaxations that were similar in extent to the nicotine-induced relaxations.

**Discussion**

We previously demonstrated that the in vivo resting tone of the clasp and sling muscle fibers is mediated in large part by neuronal release of acetylcholine acting on muscarinic receptors (Brasseur et al., 2007). We also demonstrated that the clasp/sling muscle fiber complex does not contribute to the pressure generated by the HPZ in patients with GERD as they do in normal volunteers (Miller et al., 2009). In addition, clasp fibers from organ donors with histological evidence of reflux (Barrett’s esophagitis) have a reduced contractile response compared with donors without GERD (Miller et al., 2010). These finding suggest that reflux may be the result of an abnormal clasp/sling muscle fiber complex, which could be attributed to either an underactive contractile mechanism or an overactive relaxation mechanism. The aim of the current study is to understand the mechanism of sling and clasp muscle fiber relaxation in response to activation of nicotinic receptors in the setting of contraction through activation of muscarinic receptors.

We previously reported that the bethanechol-induced maximal contraction is greater in the sling muscle fibers than in all other fibers studied in these subjects (Braverman et al., 2009). This result is in general agreement with previous studies showing that human gastric sling muscle fibers contract significantly greater to acetylcholine than human clasp muscle fibers (Tian et al., 2004). Over the span of the last 3 decades, the formation and regulation of the HPZ by the gastric sling and clasp muscle fibers have been studied in vitro. Differences have been reported between human clasp and sling stomach muscle fibers in the sensitivity and maximal responses to acetylcholine, dopamine, phenylephrine, and isoproterenol (Tian et al., 2004).

Most of the previously published studies pertaining to the relaxation of gastrointestinal smooth muscle focused on either EFS-induced relaxations or inhibition of stretch-induced active tone. EFS is known to induce effects on smooth muscle strips indirectly by inducing release of neurotransmitters from nerve endings. In the current study, TTX prevented a large portion of the nicotine-mediated relaxation of the clasp fibers. This indicates that a portion of the nicotinic-mediated relaxation of the clasp fibers is neuronally mediated and probably results from nicotine-induced stimulation of action potentials. Therefore, these nicotinic receptors must be located at sites along the nerve more proximal than the neuromuscular junction, for example on the enteric ganglion cells or axons rather than at the nerve terminals. Because TTX was ineffective in preventing nicotine-induced relaxations in sling and LEC fibers, this indicates that nicotine-induced stimulation of action potentials are not required for relaxations; thus, these nicotinic receptors are likely to be located more distally and closer to the neuromuscular junction.
With regard to the location of enteric neuronal cell bodies in the clasp, sling, and LEC regions, our immunohistochemical investigation showed that there were several small neuronal cell bodies located within the inner circular layer itself. The location of these small neurons match those described in the opossum lower gastroesophageal high-pressure zone (Sengupta et al., 1987). In that study, small groupings of "atypical" thionin-Sudan black-stained (i.e., Nissl-stained) neurons were buried within the circular muscle, in addition to groupings of neurons buried within the longitudinal muscle layer and between the longitudinal and circular layers. The latter two areas were not studied here because our focus was on the possible location of neurons within the muscle strips that were examined physiologically. Our findings in the LEC region also match those by Kim et al. (2008), who observed Pgp9.5 neuronal structures buried within muscular layers of esophageal wall of patients with esophageal myopathies. Because our and Kim’s studies are the first to examine the location of neuronal structures in the GEJ of humans using specific neuronal markers, perhaps the location of these neurons are in fact not atypical but the norm.

Neuronal NO seems to be a major neurotransmitter mediating nerve-evoked relaxations in human gastric clasp muscle fiber strips (Gonzalez et al., 2004). β-Adrenergic and D₂ dopamine receptor agonists (Tian et al., 2004) as well as vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), and adenosine have also been reported to relax human clasp muscle fibers (Gonzalez et al., 2004). The nonadrenergic, noncholinergic neurotransmitters NO, VIP, PACAP, ATP, calcitonin gene-related polypeptide (CGRP), and CO all have inhibitory effects in the gastrointestinal tract. Immunohistochemistry of the LES myenteric neurons shows the presence of NOS, the peptides VIP, PACAP, and CGRP, and the constitutive enzyme heme oxygenase type 2 that is involved in the synthesis of CO (Deman et al., 1991; Murray et al., 1991; Tøttrup et al., 1991; Oliveira et al., 1992; Tøttrup et al., 1993; Kortezova et al., 1996; Yuan et al., 1998; Farre et al., 2006; Farré et al., 2007).

The role of NO in relaxation of LES smooth muscle has been extensively studied in vitro. Stimulation of the intrinsic nerves by EFS induces an on contraction instead of an “on” relaxation in circular esophagogastric junction muscle strips from achalasic patients, suggesting that nitricergic neurotransmission is severely impaired (Tøttrup et al., 1990). In many different species, NO, NO donors, and EFS can induce relaxation and hyperpolarization of smooth muscle strips from the LES by a cGMP pathway. Experiments using cGMP inhibitors and direct measurements of cGMP confirmed that activation of nitricergic myenteric neuron induces smooth muscle relaxation via the guanylate cyclase-cGMP pathway (Torphy et al., 1986; Barnette et al., 1989; Rattan and Moumni, 1989; Conklin and Du, 1992; Jun et al., 2003; Farré et al., 2006). A current hypothesis proposes that NO inhibits calcium-activated chloride current via intracellular increase of cGMP, leading to inhibitory junction potentials and relaxation (Zhang and Paterson, 2003). Nitric oxide is considered the main inhibitory neurotransmitter at the LES.

Nicotine relaxes carbachol-precontracted human esophageal muscle strips, which are mostly blocked by L-N-nitroarginine and completely blocked by hexamethonium (Gonzalez et al., 2004). Studies using porcine clasp fibers demonstrate that nicotine indirectly relaxes smooth muscle because relaxation is inhibited by hexamethonium and TTX (Farre et al., 2006). Nicotine has been reported to stimulate two independent pathways. One is NO-mediated, and the other is sensitive to the ATP-type calcium-activated K⁺ channel blocker apamin and mediated mostly by P2Y with a minor P2X purinergic receptor contribution (Farre et al., 2006). In the current study, we demonstrated that clasp, sling, and LEC muscle strips, precontracted with bethanechol, showed relaxation with administration of nicotine and that the relaxation was blocked to variable degrees using TTX (only in clasp fibers), the NOS inhibitor

Fig. 5. Mean ± S.E.M. of the maximal contraction to 30 μM bethanechol (A, B, and C) and the maximal relaxation response (D, E, and F) to nicotine (NIC, 0.1, 0.3, and 1 mM) before (open bars) and after (shaded bars) 30-min exposure to vehicle (H₂O, Veh), 100 μM bicuculline (BIC), 100 μM L-NAME, 10 μM propranolol (Prop), 10 μM strychnine (Stry), and 1 μM tetrodotoxin (TTX). For clasp and sling fibers, all bars represent mean ± S.E.M. from at least eight muscle strips from three different specimens. For LEC fibers, n = at least six muscle strips from four specimens, with the exception of Veh (two specimens) and TTX (one specimen). Statistical difference between pre- and postresponses is indicated by ** (p < 0.01).
L-NAME, and the /H9252-adrenergic receptor antagonist propranolol. The glycine receptor antagonist strychnine and the GABA_A antagonist bicuculline inhibited relaxations with potencies consistent with nicotinic receptor blockade. Because the glycine receptor antagonist ginkgolide B had no effect on nicotine-induced relaxations and the glycine agonists taurine and glycine did not induce relaxation, we can conclude that the nicotine-induced relaxations are not mediated by activation of glycine receptors in clasp or sling fibers. The more selective GABA_A antagonist SR95531 did not affect nicotine-induced relaxations.

Fig. 6. Mean ± S.E.M. of the contraction response to bethanechol and relaxation responses to nicotine before (open bars) and after (shaded bars) 30-min exposure to vehicle (H_2O, VEH) or propranolol (1, 3, and 10 μM) for clasp fibers (top panels), sling fibers (middle panels; 0.1, 0.3, 1, 3, and 10 μM), and for LEC fibers (bottom panels; 1, 3, and 10 μM). Statistical difference between pre- and postresponses is indicated by * (p < 0.05) and ** (p < 0.01). n = 4–15 clasp muscle strips from two to four specimens, n = 4–30 sling muscle strips from two to four specimens, and n = 3–12 LEC muscle strips from two to four specimens.

Fig. 7. Concentration-response curves for bicuculline (BIC), L-NAME, propranolol (PROP), and strychnine (STRY) inhibition of 1 mM nicotine-induced relaxations of 30 μM bethanechol precontracted clasp (A), sling (B), and LEC (C) muscle fibers. The data are shown as the percentage (mean ± S.E.M.) of the relaxation response to nicotine after the addition of antagonist to the relaxation response to nicotine before the addition of antagonist calculated for each muscle strip. These curves were derived from 4–15 clasp muscle strips from two to four specimens, 4–30 sling muscle strips from two to four specimens, and 3–12 LEC muscle strips from two to four specimens for each concentration of antagonist.
that relax the clasp, sling, and LEC smooth muscles, including NO in clasp, sling, and LEC fibers, norepinephrine acting on β-adrenoceptors in clasp fibers, and GABA acting on GABA_A receptors in sling fibers. Because selective relaxation of these particular smooth muscle fibers of the GEJ are early events in the TLESRs that mediate GERD, identification of agents that selectively prevent these relaxations may be useful therapeutic agents to treat GERD by preventing TLESRs.

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Authorship Contributions
Participated in research design: Braverman, Vegesna, Miller, and Ruggieri.
Conducted experiments: Braverman, Vegesna, Barbe, Tiwana, Hussain, and Ruggieri.
Contributed new reagents or analytic tools: Barbe.
Performed data analysis: Braverman, Vegesna, and Ruggieri.
Wrote or contributed to the writing of the manuscript: Braverman, Vegesna, Miller, and Ruggieri.

References
Bettarello A, Tuttle SG, and Grossman MI (1960) Effects of Autonomic Drugs on Neuronal nicotinic receptors modulate synaptic function in the hippocampus and are sensitive to blockade by the convulsant strychnine and by the anti-Parkinson drug amantadine. Toxicol Lett 102–103:211–218.

TABLE 1

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Clasp IC_50 (µM)</th>
<th>Sling IC_50 (µM)</th>
<th>LEC IC_50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicucculine</td>
<td>38.5 ± 3.4</td>
<td>47.6 ± 7.4</td>
<td>85 ± 9.1</td>
</tr>
<tr>
<td>t-NAME</td>
<td>24.7 ± 6.0</td>
<td>7.9 ± 1.2</td>
<td>102 ± 94</td>
</tr>
<tr>
<td>Propranolol</td>
<td>6.1 ± 2.1</td>
<td>1.27 ± 0.47*</td>
<td>1.83 ± 1.1</td>
</tr>
<tr>
<td>Strychnine</td>
<td>9.0 ± 1.1</td>
<td>28.2 ± 17</td>
<td>44.9 ± 28</td>
</tr>
</tbody>
</table>

The fact that TTX blocks the nicotine-mediated relaxation in clasp fibers suggests that nicotine causes the relaxation early on in the pathway at a neuromuscular junctional site. Historically, NO has been thought to be the final mediator of relaxation. However, NOS inhibition did not completely block the relaxation, suggesting that other non-nitricergic neurotransmitters are also involved. The nitric oxide donor SNAP caused the same degree of relaxation in both clasp and sling fibers as nicotine. The β-adrenoceptor agonist isoproterenol relaxed clasp fibers nearly as much as nicotine but did not relax sling fibers, whereas the GABA_A agonist muscimol did not relax either clasp or sling fibers. It is not entirely clear how the β-adrenoceptor antagonist propranolol can completely prevent the nicotine-induced relaxations in both clasp and sling fibers, whereas the β-adrenoceptor agonist isoproterenol only relaxes the clasp and not sling fibers. Likewise, it is unclear how the GABA_A antagonist SR95531 can inhibit nicotine-induced relaxations in sling fibers by 35%, whereas the relaxation effect of the GABA_A agonist muscimol was not evident in sling fibers. One possible explanation for these findings is that a threshold concentration of each of the neurotransmitters (norepinephrine and GABA) is needed to induce a relaxation. Therefore, any one of the individual antagonists could prevent the relaxations. A second explanation is that the neural pathways involved in relaxation are connected in series rather than in parallel. Therefore, blocking one neurotransmitter blocks the action of the other neurotransmitter and effectively blocks the relaxation.

Based on the findings of this study, we can make the conclusions that, in the face of muscarinic receptor stimulation, activation of nicotinic receptors on the enteric neurons in GEJ smooth muscle causes release of multiple substances...


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Supplemental Figure 1. Representative traces of sling fiber contractile responses to bethanechol, the relaxation response to nicotine and the inhibitory effects of bicuculline, L-NAME, propranolol and strychnine on the nicotine induced relaxations.
Supplemental Figure 2. Representative traces of LEC fiber contractile responses to bethanechol, the relaxation response to nicotine and the inhibitory effects of bicuculline, L-NAME, propranolol and strychnine on the nicotine induced relaxations.
Supplemental Figure 3. Relaxation responses of clasp fibers to nicotine (0.1, 0.3, and 1 mM) pre-contracted with 30 μM bethanechol before and after exposure to vehicle (H2O), 100 μM bicuculline, 10 μM L-NAME, 10 μM propranolol, 33 μM strychnine and 1 μM tetrodotoxin. The data is presented as the % relaxation of the bethanechol induced maximum tension. All data points are derived from 3-5 muscle strips from at least 2 specimens, except strychnine (n=2 strips from 1 specimen). Statistically significant differences between pre and post antagonist exposure is indicated by * (p<0.05) and ** (p<0.01).
Supplemental Figure 4. Relaxation responses of sling fibers to nicotine (0.1, 0.3, and 1 mM) pre-contracted with 30 μM bethanochol before and after exposure to vehicle (H2O), 100 μM bicuculline, 10 μM L-NAME, 10 μM propranolol, 33 μM strychnine and 1 μM tetrodotoxin. The data is presented as the % relaxation of the bethanochol induced maximum tension. All data points are derived from at least 9 muscle strips from at least 2 specimens. Statistically significant differences are indicated as in supplemental figure 3.
Supplemental Figure 5. Relaxation responses of LEC fibers to nicotine (0.1, 0.3, and 1 mM) pre-contracted with 30 μM bethanechol before and after exposure to vehicle (H2O), 100 μM bicuculline, 10 μM LNAME, 10 μM propranolol, 33 μM strychnine and 1 μM tetrodotoxin. The data is presented as the % relaxation of the bethanechol induced maximum tension. All data points are derived from at least 4 muscle strips from 1 specimen. Statistically significant differences are indicated as in supplemental figure 3.
**Supplemental Figure 6.** Mean ± SEM of the contractile response to 30 μM bethanechol (A, C and E) and the relaxation response to 1 mM nicotine following bethanechol contraction (B, D and F) of clasp fibers before (open bars) and after (shaded bars) 30 minute exposure to vehicle (H2O), bicuculline (A and B), L-NAME (C and D) and strychnine (E and F). n=4-15 clasp muscle strips from 2-4 specimens. Statistically significant differences are indicated as in supplemental figure 3.
Supplemental Figure 7. Mean ± SEM of the contraction to 30 μM betahanechol (A, C and E) and the relaxation response to 1 mM nicotine (B, D and F) of sling fibers to nicotine before (open bars) and after (shaded bars) 30 minute exposure to vehicle (H2O), bicuculine (A and B), L-NAME (C and D) and strychnine (E and F). n=4-30 sling muscle strips from 2-4 specimens. Statistically significant differences are indicated as in supplemental figure 3.