Variability in the muscle composition of rat esophagus and neural pathway of lower esophageal sphincter relaxation

Yanfen Jiang, Valmik Bhargava, Harshal A. Lal, and Ravinder K. Mittal

Division of Gastroenterology, Department of Medicine, San Diego Veterans Affairs Health Care System & University of California, San Diego, California

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Jiang Y, Bhargava V, Lal HA, Mittal RK. Variability in the muscle composition of rat esophagus and neural pathway of lower esophageal sphincter relaxation. *Am J Physiol Gastrointest Liver Physiol* 301: G1014–G1019, 2011. First published September 1, 2011; doi:10.1152/ajpgi.00273.2011.—Several studies from our laboratory show that axial stretch of the lower esophageal sphincter (LES) in an oral direction causes neurally mediated LES relaxation. Under physiological conditions, axial stretch of the LES is caused by longitudinal muscle contraction (LMC) of the esophagus. Because longitudinal muscle is composed of skeletal muscle in mice, vagal-induced LMC and LES relaxation are both blocked by pancuronium. We conducted studies in rats (thought to have skeletal muscle esophagus) to determine if vagus nerve-mediated LES relaxation is also blocked by pancuronium. LMC-mediated axial stretch on the LES was monitored using piezoelectric crystals. LES and esophageal pressures were monitored with a 2.5-Fr solid-state pressure transducer catheter. Following bilateral cervical vagotomy, the vagus nerve was stimulated electrically. LES, along with the esophagus, was harvested after in vivo experiments and immunostained for smooth muscle (smooth muscle α-actin) and skeletal muscle (fast myosin heavy chain). Vagus nerve-stimulated LES relaxation and esophageal LMC were reduced in a dose-dependent fashion and completely abolished by pancuronium (96 μg/kg) in six rats (group 1). On the other hand, in seven rats, LES relaxation and LMC were only blocked completely by a combination of pancuronium (group 2) and hexamethonium. Immunostaining revealed that the longitudinal muscle layer was composed of predominantly skeletal muscle in the group 1 rats. On the other hand, the longitudinal muscle layer of group 2 rats contained a significant amount of smooth muscle (P < 0.05). Our study shows tight coupling between axial stretch on the LES and relaxation of the LES, which suggests a cause and effect relationship between the two. We propose that the vagus nerve fibers that cause LMC induce LES relaxation through the stretch-sensitive activation of inhibitory motor neurons.

METHOD

Studies were performed in 13 Sprague Dawley male rats weighing 250–400 g (Charles River, Wilmington, MA). The animal safety committee of the San Diego Veterans Affairs Health Care System approved the study protocol. Animals were anesthetized with an intraperitoneal injection of a stock solution containing ketamine (3.75 ml, 100 mg/ml), xylazine (0.4 ml, 100 mg/ml), acepromazine (0.75 ml, 10 mg/ml), and 15.1 ml distilled water. For induction of anesthesia, 4 ml/kg of the above solution were injected intraperitoneally. In addition, 0.8 ml of the solution was injected intermittently as needed for maintenance of anesthesia. Adequate depth of anesthesia was monitored by the absence of toe pinch reflex. Following administration of pancuronium, anesthesia was maintained by injection of maintenance doses every 45 min. A venous cannula was placed in the internal jugular vein (for administration of pharmacological agents), an arterial line was placed in the carotid artery (to measure blood pressure), and a tracheotomy was performed for ventilation (85 strokes/min, tidal volume of 2 ml) using a Harvard pump (model no. 683; Holliston). The vagus nerve was isolated on both sides in the neck and transected; the peripheral end of one of the vagus nerves was placed on a pair of platinum electrodes for electrical stimulation using a dual-output square pulse generator (S88X; Grass, West Warwick,
The electrostimulus frequency was 2, 5, 10, 20, and 30 Hz, with a pulse amplitude of 10 V, pulse width of 10 ms, and a train duration of 5 s. A midline laparotomy was performed, and the esophagogastroduodenal junction was identified. Longitudinal muscle contraction/axial stretch on the LES was measured by two 2-mm piezoelectric crystals (Sonometrics; London, Ontario, Canada). One crystal (moving crystal) was anchored on the upper edge of the LES with a 2% glutaraldehyde solution. The other crystal (fixed crystal) was mounted on a wooden stick and placed in the abdomen close to the LES (9). Circular muscle contraction of the esophagus was measured with a 2.5-Fr solid-state pressure transducer catheter equipped with four sensors (custom designed by Millar Instruments, Houston, TX). The catheter was passed through a small incision on the stomach, 2 cm below the LES. Two pressure transducers, spaced 0.55 mm apart, were placed in the LES (high-pressure area), and the other two were located in the esophagus 15 and 30 mm above the LES (Fig. 1). Tissue adhesive (NEXABAND S/C Topical Tissue Adhesive, Closure Medical, distributed by Abbott, North Chicago, IL) was used to glue the catheter to the stomach wall to avoid relative movement between the LES and the esophageal pressure transducer (Fig. 1).

**Functional study.** Physiological data were recorded during the following time periods: 1) control period: LES pressure, esophageal pressure, and longitudinal muscles contraction (axial stretch on the LES) were recorded during vagal stimulation at the above-mentioned frequencies; 2) pancuronium was administered intravenously at increasing concentrations (12, 24, 48, 96, and 192 μg/kg), and, after each dose, vagus nerve stimulation was repeated at all frequencies; and 3) in those animals where vagus nerve-stimulated LES relaxation was not completely abolished (>95%) by the highest dose of pancuronium, hexamethonium (20 mg/kg) was administered intravenously, followed by electrical stimulation of the vagus nerve, as in the control period.

At the end of each experiment, the esophagus, LES, and part of the stomach were harvested, followed by an injection of euthanasia solution to the animal (potassium chloride solution, 2 mM/kg). This euthanasia procedure is in accordance with the guidelines of the San Diego Veterans Affairs Animal Committee.

**Immunohistochemistry of esophageal wall.** Harvested esophagi along with the stomach was fixed at the original length in FORMAL FIXX for 4 h, transferred to 75% alcohol, and embedded in paraffin. Tissue samples were sectioned in 10 μm thickness. Sections were labeled with primary rabbit anti-smooth muscle α-actin (ab5694; abcam, Cambridge, MA) and mouse fast myosin skeletal muscle heavy chain (ab51263; abcam) antibodies, each at 1:250 concentrations. Incubations were performed either overnight at 4°C or for 1 h at room temperature. Negative controls were performed by omitting the primary antibodies during the staining process. Secondary antibodies used were as follows: 1) goat anti-rabbit DyLight 488-conjugated and 2) goat anti-mouse DyLight 549-conjugated IgG (H+L) each at 1:500 concentration (from Jackson Immuno Research Laboratories, West Grove, PA). A mounting medium containing DAPI along with a cover slip was placed on the slides. Sections were viewed with a fluorescent microscope (Nikon Ti, Melville, NY).

**Data analysis.** LES pressure was measured as end-expiratory pressure above gastric pressure, and percent LES relaxation was calculated. The change in distance between the two piezoelectric crystals (crystal length) was recorded as a measure of longitudinal muscle contraction-induced cranial displacement of the LES. Thus, the effects of vagus nerve stimulation on esophageal circular muscle contraction, LES pressure, and LES cranial movement (crystal length) were determined.

**Image analysis.** From the immunostained color images, blue color (nuclear stain) was suppressed. With the use of Adobe Photoshop 7.0.1, the threshold value as determined by each respective negative control was set to zero. For smooth muscle (seen as green color) and skeletal muscle (seen as red color), all pixel values above the threshold were set to a full brightness of 255. The average green or red intensity from the adjusted image was measured using Nikon’s NIS-Elements AR 3.10 program. The number of pixels that were set to 255 for green color was calculated as: 
\[ N_g = \frac{T \times A_g \times 100}{255} \]
where \( N_g \) is the number of green pixels above the threshold value, \( T \) is the total number of pixels in the region of interest, and \( A_g \) is the average green intensity. The number of red pixels and the average were calculated in the same way from the total number of pixels and the average red intensity.

**Statistical analysis.** The differences between two groups of rats were determined by the unpaired Man Whitney U-test. The differences before and after hexamethonium were determined by the Wilcoxon matched-pairs signed-rank test. Multiple comparisons were done using Tukey’s test. Data are presented as means ± SE. P values <0.05 were considered statistically significant.

**RESULTS**

**Effect of vagus nerve stimulation on LES pressure, LES cranial displacement, and esophageal contractions.** Electrical stimulation of the peripheral end of the cervical vagus nerve induced a frequency-dependent increase in the amplitude of esophageal circular muscle contraction, longitudinal muscle contraction (seen as cranial displacement of the LES), and relaxation of the LES. The duration of electrical stimulation was similar to the durations of LES relaxation and esophageal circular and longitudinal muscle contraction (Figs. 2–4).

**Effect of pancuronium on LES relaxation, LES cranial displacement, and esophageal contractions.** The vagus nerve was stimulated at maximal frequency (30 Hz), and the effects of different doses of pancuronium were studied during these experiments. Esophageal circular muscle contraction was reduced in a dose-dependent fashion by pancuronium and at a dose of 96 μg/kg was almost completely abolished in each of the rats studied. On the other hand, effects of pancuronium on the longitudinal muscle contraction and LES relaxation were variable. LES relaxation was completely blocked (>95%) in six rats, and these were labeled as group 1 animals. On the other hand, block of LES relaxation by pancuronium in seven rats was significantly less, 90 ± 4% in the control period to 46 ± 7% after the highest dose of pancuronium. These rats were labeled as group 2 animals. In the group 1 animals, LES cranial displacement was abolished almost completely, following 96 μg/kg doses of pancuronium (1.42 ± 0.12 mm in the control period to 0.015 ± 0.01 mm after pancuronium). On the other hand, in group 2 rats, pancuronium (96 μg/kg) only partially blocked the vagal nerve-stimulated cranial displacement of LES (from 1.91 ± 0.47 mm in the control period to 0.34 ± 0.13 mm after the largest dose of pancu-
Craniocranial displacement after administration of pancuronium in the two groups is significantly different (Mann-Whitney test, \( P < 0.05 \)).

**Effect of hexamethonium on vagus nerve-stimulated LES relaxation and LES cranial displacement.** In group 2 animals, cranial displacement of LES and LES relaxation were still present after 192 \( \mu \)g/kg of pancuronium, 0.17 ± 0.02 mm and 35 ± 9%, respectively. Hexamethonium, 20 mg/kg, was administered after the last dose of pancuronium, which resulted in a complete abolishment of both residual LES cranial displacement and LES relaxation, 3 ± 1% and 0.02 ± 0.01 mm, respectively (Figs. 2, 3, and 6). The differences before and after hexamethonium for cranial displacement and LES relaxation are significant (Wilcoxon matched-pairs signed-rank test, \( P < 0.05 \)).

**Fig. 2.** Effect of vagus nerve stimulation on the longitudinal muscle contraction (cranial displacement of the LES, crystal length), circular muscle contraction (esophageal contraction), and LES relaxation. Pancuronium abolished all responses induced by vagus nerve stimulation. Vagal stimulation parameters were frequency 2, 5, 10, 20, and 30 Hz; pulse amplitude 10 V; pulse width 10 ms; and pulse duration 5 s.

**Fig. 3.** Effect of vagus nerve stimulation on the longitudinal muscle contraction (cranial displacement of LES, crystal length), circular muscle contraction (esophageal contraction), and LES relaxation. Only esophageal contraction (circular muscle) is completely blocked by pancuronium; longitudinal muscle contraction and LES relaxation are completely blocked by addition of 20 mg/kg of hexamethonium after pancuronium. Vagal stimulation parameters were frequency 2, 5, 10, 20, and 30 Hz; pulse amplitude 10 V; pulse width 10 ms; and pulse duration 5 s.

**Fig. 4.** Summary data of the effects of different frequency and pancuronium on vagus nerve-stimulated longitudinal muscle contraction (cranial displacement of LES recorded by crystal length), circular muscle contraction (recorded by intraluminal pressure), and LES relaxation. Data are shown as means ± SE; \( n = 13 \) in group 1 and \( n = 6 \) in group 2. The effect of pancuronium as a function of stimulation frequency is compared with control. The difference is significantly different by Tukey’s Multiple Comparison Test. \(* P < 0.05 \) and \( ** P < 0.01 \).
Immunohistochemistry of esophageal wall. Under the fluorescent microscope, smooth muscle α-actin and skeletal muscle fast myosin heavy chain were visualized as green and red color, respectively. Muscularis propria of rat esophagus is organized into two layers, inner circular and outer longitudinal muscle. The inner circular muscle coat was made up of mostly skeletal muscles in both group 1 and group 2 rats. On the other hand, there were differences in the compositions of smooth and skeletal muscles in the longitudinal muscle layer in the two groups. In group 1 animals, the longitudinal muscle layer is made of mostly skeletal muscles. Even though some green color was seen in the longitudinal muscle layer in group 1 rats, it appeared to be organized in the circular shape and was most likely located in the blood vessels. On the other hand, in the group 2 animals, bundles of smooth muscle that extended for several millimeters in the cranial direction were seen in the longitudinal muscle layers. There was a statistically significant difference ($P = 0.005$, Man-Whitney test) in the green immune fluorescence between group 1 (36 ± 3%) and group 2 animals (24 ± 2%) (Fig. 7).

DISCUSSION

In summary, our data show variability in the effects of pancuronium or skeletal muscle paralysis on the vagus nerve-induced LES relaxation in rats. While in some rats LES relaxation is completely blocked by pancuronium, in others ganglionic block with hexamethonium was required. Most interesting, we found that, in those rats where vagus nerve-stimulated LES relaxation is completely blocked by pancuronium, the longitudinal muscle layer of the esophagus is composed of mostly skeletal muscles. On the other hand, group 2 rats, which required hexamethonium to completely block vagus nerve-stimulated LES relaxation, contained a significantly large amount of smooth muscle in the longitudinal muscle layer compared with group 1 rats. Longitudinal muscle contraction and LES relaxation appear to be tightly linked: complete inhibition of longitudinal muscle contraction and block of LES relaxation occur together. We propose that the vagus nerve-mediated LES relaxation is mediated through the longitudinal muscle contraction-induced activation of mechanosensitive inhibitory motor neurons of the LES.

Human and animal studies show that LES relaxation associated with swallow (3), balloon distension of the esophagus (19), and electrical stimulation of the vagus nerve (4) is associated with cranial displacement of the LES. The latter is the result of the contraction of the longitudinal muscle layer of the esophagus. Similarly, transient LES relaxation, the major mechanism of gastroesophageal reflux, such as belching and vomiting, is associated with localized contraction of the longitudinal muscle of the distal esophagus (1) that results in cranial displacement of the LES (17). Our hypothesis is that a cranial axial stretch on the LES activates inhibitory motor neurons and consequently results in relaxation of the LES. These inhibitory motor neurons are located in the myenteric plexus of the esophageal wall. Accordingly, we found that a mechanical pull of the LES in the cranial direction induces neurally mediated LES relaxation in opossum (5). Our detailed studies on the mechanism of axial stretch-activated LES relaxation in mice show that there is no neural synapse involved in this neural reflex (9). In mice with a skeletal muscle esophagus and smooth muscle LES, we found that pancuronium abolished both, i.e., longitudinal muscle contraction of esophagus and LES relaxation induced by vagus nerve stimulation (9). In another study in rats, we observed that fundoplication (a surgical procedure to treat reflux disease) restricts vagus nerve-stimulated axial stretch on the LES and blocks LES relaxation (10). We were initially surprised at our observation that pancuronium did not completely block vagus nerve-induced LES relaxation in rats because the esophagus in rats is thought to be primarily made of skeletal muscle (25). Our current study revealed that, in those animals where LES relaxation was not blocked by pancuronium, longitudinal muscle contraction of

Fig. 5. Summary of the effect of pancuronium on cranial displacement of LES (crystal length, left), esophageal contraction (right), and LES relaxation (middle) during vagus nerve stimulation. Vagus nerve stimulation frequency was 30 Hz, pulse amplitude 10 V, pulse width 10 ms, and train duration 5 s. Pancuronium doses were 12, 24, 48, and 96 μg/kg; ●, group 1 (G1, n = 6); ○, group 2 (G2, n = 7). All data are means ± SE. *$P < 0.05$. Differences between two groups are significantly different by Wilcoxon matched-pairs signed-rank test, $P < 0.05$.

Fig. 6. Summary of the effect of hexamethonium on cranial displacement of LES (crystal length, left) and LES relaxation (right): vagus nerve stimulation frequency 30 Hz; pulse amplitude 10 V; pulse width 10 ms, and train duration 5 s in the presence of 192 μg/kg pancuronium and 20 mg/kg hexamethonium. Pancuronium plus hexamethonium totally blocked the vagus nerve stimulation-induced longitudinal muscle contraction and LES relaxation. ***$P < 0.001$. Differences between 2 groups are significantly different by Wilcoxon matched-pairs signed-rank test, $P < 0.05$.

Fig. 7. Immunohistochemistry of esophageal wall. Under the fluorescent microscope, smooth muscle α-actin and skeletal muscle fast myosin heavy chain were visualized as green and red color, respectively. Muscularis propria of rat esophagus is organized into two layers, inner circular and outer longitudinal muscle. The inner circular muscle coat was made up of mostly skeletal muscles in both group 1 and group 2 rats. On the other hand, there were differences in the compositions of smooth and skeletal muscles in the longitudinal muscle layer in the two groups. In group 1 animals, the longitudinal muscle layer is made of mostly skeletal muscles. Even though some green color was seen in the longitudinal muscle layer in group 1 rats, it appeared to be organized in the circular shape and was most likely located in the blood vessels. On the other hand, in the group 2 animals, bundles of smooth muscle that extended for several millimeters in the cranial direction were seen in the longitudinal muscle layers. There was a statistically significant difference ($P = 0.005$, Man-Whitney test) in the green immune fluorescence between group 1 (36 ± 3%) and group 2 animals (24 ± 2%) (Fig. 7).

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the esophagus was also not completely blocked. Immunohistochemistry of the esophageal wall, for skeletal and smooth muscles, revealed that group 2 rats contained a significantly larger amount of smooth muscle in the longitudinal or outer muscle layer compared with group 1 rats. Some of the smooth muscle was located in the vascular structures, but other was clearly present in the longitudinal muscle layer of the muscularis propria. Interestingly, the inner circular muscle layer contained mostly skeletal muscle in both groups. The reason why some animals contain smooth muscles in the longitudinal muscle coat is not clear. Studies in mice show that early age embryo esophagus is made up of all smooth muscle, which transdifferentiate into skeletal muscle at a later time (18, 20, 21). At the time of birth, the entire esophagus (not LES) is made up of skeletal muscles. It is possible that a small amount of smooth muscle in the longitudinal muscle layer is a hangover, resulting from the failure of complete transdifferentiation.

Neural mechanisms of skeletal and smooth muscle contractions are quite different. Vagal efferent nerve fibers synapse with the motor end plates of skeletal muscles (11, 25). Upon activation, vagal fibers release acetylcholine at presynaptic nerve terminals, which stimulate nicotinic cholinergic receptors on the motor end plate. Curare and “curare-like drugs” (pancuronium) are specific competitive antagonists of nicotinic receptors on the motor end plate and therefore cause paralysis of skeletal muscles. On the other hand, in the case of smooth muscle, there are no recognizable motor end plates. It is suggested that vagal efferent fibers do not innervate the end organs or the smooth muscle directly. Instead, they synapse with motor neurons located in the myenteric plexus (2), which in turn release excitatory or inhibitory neurotransmitters, i.e., acetylcholine and substance P to cause contraction of the smooth muscle and nitric oxide to cause relaxation. Synapses between the vagus nerve and myenteric neurons are mostly cholinergic (7), and hexamethonium is a specific blocker of the cholinergic neural synapse. Accordingly, we found that hexamethonium abolished the residual longitudinal muscle contraction after pancuronium administration.

Pancuronium abolished longitudinal muscle contraction and LES relaxation in group 1 rats. On the other hand, in group 2 rats, both of the above parameters were incompletely blocked by pancuronium. We observed a close correlation between residual longitudinal muscle contraction and residual LES relaxation after pancuronium, raising the possibility of a cause-and-effect relationship between the axial stretch on the LES and LES relaxation. The myenteric plexus contains predominantly two types of motor neurons, excitatory and inhibitory. These can be recognized by immunohistochemistry; acetylcholine and substance P are present in the excitatory motor neurons and nitric oxide in the inhibitory motor neurons (6). Current thinking is that the vagus nerve contains two types of efferent fibers, which run in parallel; i.e., one kind synapses with excitatory motor neurons and the other with the inhibitory motor neurons (2). However, there is no direct demonstration of the above. It is possible that all vagus nerve fibers are excitatory and the ones that innervate longitudinal muscles activate inhibitory motor neurons through a stretch-sensitive mechanism. Recent studies show that large numbers of neurons in the myenteric plexus of the small intestine and colon have mechanosensitive properties (23, 24). It is possible that “motor neurons” have a dual function, i.e., they can sense as well as release neurotransmitters to cause muscle contraction or relaxation. Based on our previous studies and current observation of tight coupling between cranial LES displacement and LES relaxation, we postulate that the variability in muscle compo-

![Fig. 7. Immunostaining of esophageal wall: smooth muscle α-actin (green), skeletal muscle heavy chain (red), and DAPI for nucleus (blue) stains. Left, low-magnification (×4) coronal section including LES and stomach; middle, cross section just above LES (×10); right, magnified coronal section (×40). LM, longitudinal muscle; CM, circular muscle; MM, muscularis mucosa. Note, there is a significant amount of smooth muscle in the longitudinal muscle layer of the group 2 rats. On the other hand, in the group 1 rats, the longitudinal muscle layer was composed of skeletal muscles only.](http://ajpgi.physiology.org)
sition of the esophagus, i.e., smooth vs. skeletal, explains the variability in the mechanism of vagus nerve-induced LES relaxation. In those animals where the esophagus is made up of mostly skeletal muscle fibers, vagus nerve-stimulated LES relaxation is blocked completely by pancuronium. On the other hand, animals with a smooth muscle esophagus require a ganglionic blocker and block of longitudinal muscle contraction to antagonize vagus nerve-stimulated LES relaxation. In opossum, with predominant smooth muscle esophagus (same as human), a combination of hexamethonium and atropine is required to completely block vagus nerve-stimulated LES relaxation, which is the basis of the current understanding that nicotinic and muscarinic receptors are involved at the postsynaptic site on the inhibitory motor neuron. However, the possibility that the vagus nerve actually activates longitudinal muscle contraction, and the axial stretch caused by longitudinal muscle contraction induces LES relaxation, cannot be excluded from these studies. Future studies are needed to investigate the precise neural pathway of vagus nerve-mediated LES relaxation in the opossum because, similar to humans, they have a smooth muscle distal esophagus.

REFERENCES