Evidence for a peripheral mechanism of esophagocrural diaphragm inhibitory reflex in cats

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Liu, Jianmin, Yoshihiro Yamamoto, Bruce D. Schirmer, Robert A. Ross, and Ravinder K. Mittal. Evidence for a peripheral mechanism of esophagocrural diaphragm inhibitory reflex in cats. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G281–G288, 2000.—The esophagogastric junction (EGJ) is guarded by two sphincters, a smooth muscle lower esophageal sphincter (LES) and a skeletal muscle crural diaphragm. These two sphincters relax simultaneously under certain physiological conditions, i.e., swallowing, belching, vomiting, transient LES relaxation, and esophageal distension. Esophageal distension-induced crural diaphragm relaxation is mediated through vagal afferents that are thought to exert inhibitory influence on the central mechanism (brain stem) of crural diaphragm contraction. We conducted studies in 10 cats to determine whether a mechanism of crural diaphragm relaxation was located at the level of the neuromuscular junction and/or muscle. Stimulation of the crural diaphragm neuromuscular junction through 1) the electrodes implanted in the muscle and 2) the bilateral phrenic nerve resulted in an increase in EGJ pressure. Nicotinic receptor blockade (pancuronium, 0.2 mg/kg) abolished the EGJ pressure increase caused by electrical stimulation of the neuromuscular junction. Esophageal distension and bolus-induced secondary esophageal peristalsis caused relaxation of the EGJ during the stimulation of the neuromuscular junction. Bilateral phrenicotomy and vagotomy had no influence on this relaxation. These data suggest the existence of a peripheral mechanism of crural diaphragm inhibition. This peripheral inhibitory mechanism may reside at the level of either the neuromuscular junction or the skeletal muscle.

The esophagogastric junction (EGJ), in animals as well as humans, is guarded by two sphincters, a smooth muscle lower esophageal sphincter (LES) and a skeletal muscle crural diaphragm (13). In humans and cats, under normal conditions, these two sphincters are anatomically superimposed on each other (5, 12). The end-expiratory EGJ pressure in the resting state is caused by the contraction of the LES, and the increase in EGJ pressure due to inspiration is caused by crural diaphragm contraction (6, 22). These two sphincters, LES and crural diaphragm, relax simultaneously under certain physiological conditions, i.e., swallowing, belching, and vomiting (2, 23, 31). Transient LES relaxation, the major mechanism of gastroesophageal reflux, is also accompanied by simultaneous relaxation of the LES and crural diaphragm (17, 20, 21).

The smooth muscle LES relaxation during a swallow and esophageal distension has been studied extensively and is mediated through both a central (brain stem and vagus nerve) and a peripheral (myenteric neurons) mechanism (18, 28). On the other hand, the mechanism mediating relaxation of the crural diaphragm is not well understood. Crural diaphragm contraction is mediated by oscillatory activity of the inspiratory neurons located in the respiratory center of the brain stem (11, 29). The activity of these neurons is transmitted to the phrenic nerve nucleus located in the cervical spinal cord, which in turn communicates through the phrenic nerve and neuromuscular junction to mediate crural diaphragm contraction. Several investigators have reported that in cats, dogs, and sheep (1, 7, 8, 14, 23, 26, 31), esophageal distension causes inhibition of the crural diaphragm. The afferent pathway for this esophagealcrural diaphragmatic inhibitory reflex is thought to be located in the vagus nerve (1, 7, 14), because bilateral vagotomy abolishes this reflex. It has therefore been suggested that the vagal afferents exert inhibitory influence on the brain stem inspiratory motor neurons that supply the crural diaphragm. However, a study by Altschuler et al. (3) did not find inhibition of medullary inspiratory neurons in response to esophageal distension in cats. Oyer et al. (27) found that in response to esophageal distension crural diaphragm electromyogram (EMG) showed complete inhibition, but there was only partial inhibition of the efferent discharge in the phrenic nerve branches to the crural diaphragm, which indicates that there may be an inhibitory mechanism located distal to the phrenic nerve. Thus the goals of our current studies were to determine whether there is an inhibitory mechanism at the level of crural diaphragm neuromuscular junction and/or skeletal muscle.

METHODS

These studies were conducted in 10 adult cats of either sex, weighing between 3 and 5 kg. The protocol for the study was approved by the Animal Ethics Committee of the University of Virginia. Two protocols were used for these experiments, protocol 1 (animals with chronically implanted electrodes) and protocol 2 (acute experiments).

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Protocol 1. A total of four cats were implanted with two pairs of platinum electrodes in the crural diaphragm. Animals were intubated, and sterile surgery was performed under general anesthesia with halothane and pentobarbital sodium. Surgery was performed either through the abdomen or through the chest for implantation of the electrodes. In two animals, the abdomen was opened through a midline laparotomy incision and the crural diaphragm was exposed on the left side, under the spleen. Each electrode consisted of a pair of platinum needles 0.6 mm in diameter and 4 mm in length. Each pair of needle electrodes, spaced 3 mm apart, was mounted in a separate silicone case. The electrodes were implanted on the esophageal hiatus (crural diaphragm) as close to the esophagus as possible. One pair was implanted on the left side of the esophagus and the other on the right side. These two sets of electrodes were connected to a cannula with insulated wires 1 mm in diameter. These wires were tunneled under the skin, and the cannula was implanted on the back of the neck. In the other two animals, the electrodes were implanted through a thoracotomy. Crural diaphragm muscle was easily accessed and more prominent from the thoracic side. The thoracotomy was performed through the eighth intercostal space. The animals were allowed to recover from the surgery, and the physiological experiments were performed 7–10 days after the surgery.

Experiments in protocol 1. Animals were lightly anesthetized using intraperitoneal injection of ketamine (25 mg/kg). An intravenous line was established for maintenance of anesthesia using intermittent bolus injections of pentobarbital sodium. Animals were placed on a heating pad for maintenance of body temperature. A manometric catheter was placed through the mouth into the esophagus and stomach for measurement of pressures. The manometry catheter was equipped with a 6-cm-long reverse-perfused sleeve (30) to measure EGJ pressure and side holes to measure pressures in the stomach (2 cm below EGJ) and esophagus at 2, 4, and 6 cm above the EGJ. Each of the side holes and the sleeve device were perfused with bubble-free water at a rate of 0.5 ml/min using a pneumohydraulic infusion system.

Electrical stimulation of the crural diaphragm was performed using a pulse generator and a constant-current stimulus unit (World Precision Instruments, New Haven, CT). An electrical stimulus of 50 Hz and 1-ms pulse width was used to stimulate the crural diaphragm. The current intensity used was varied from 1 to 2, 5, 10, 15, and 20 mA. The stimulus was delivered for periods of 10–30 s. We tested each electrode pair individually and then one pin from each pair of the electrodes to determine the best EGJ pressure response.

A short-acting neuromuscular junction-blocking drug (pancuronium, 0.2 mg/kg iv) was used to determine whether the crural diaphragm contraction induced by electrical stimulation was mediated through the neuromuscular junction or direct muscle excitation. Animals were intubated intratracheally via the mouth for these experiments.

Esophageal distension was performed using a 2-cm-long balloon placed 4–5 cm above the sleeve device. The balloon was inflated to induce relaxation of the EGJ. Balloon volumes of 2–15 ml were tested, and the volume required to induce a complete EGJ relaxation (≥80%) in the absence of crural diaphragm stimulation was determined. Each inflation of the balloon lasted 10 s and was repeated three times at 1- to 2-min intervals. Secondary esophageal peristalsis was induced by injection of 10 ml of water in the esophagus at 10 cm above the EGJ. Under each study condition, five to six peristaltic contractions were induced. Esophageal distension and secondary peristalsis were tested in the absence of crural diaphragm stimulation as control and then during crural diaphragm stimulation.

We also performed synchronized fluororomanometric studies in two cats to determine the EGJ relaxation and transit of bolus across the EGJ during control and crural diaphragm stimulation periods. The animals were positioned supine under a fluoroscopic unit to visualize the middle and distal esophagus along with a part of the stomach. During sustained crural diaphragm stimulation, a 10-ml bolus of diluted barium was injected into the proximal esophagus to induce secondary peristalsis. The fluoroscopic images were videotaped on a Sony Betamax videotape recorder, and pressures were recorded on a Sensormedics eight-channel recorder. The pressure and the videotape recordings were synchronized using a video timer (Thalaner Electronics Labs, Ann Arbor, MI).

Protocol 2. These acute experiments were performed in six cats. After intratracheal intubation, a thoracotomy was performed on the left side first followed by a thoracotomy on the right side. The phrenic nerve was identified on each side and sectioned (Fig. 1). The peripheral end of both phrenic nerves was placed in the cuff electrodes. The chest was closed after the procedure, and the wires from the electrodes were connected to the stimulator. The peripheral end of both phrenic nerves was stimulated simultaneously to induce diaphragmatic contraction. The electrical stimulus used for these experiments was a 50-Hz square wave with a positive pulse width of 1 ms. The current intensity used was 2 mA. Esophageal distensions were performed to induce EGJ relaxation in the absence of and then during phrenic nerve stimulation. In two cats, right and left vagus nerves were identified in the neck and sectioned.

Data analysis. EGJ pressure was measured as the end-expiratory pressure above the intragastric pressure. The peak EGJ pressure during crural diaphragm stimulation was measured in reference to the stomach pressure. EGJ relaxation during balloon distension and esophageal peristalsis was also measured in reference to the intragastric pressure and percent EGJ relaxation was calculated. The motion of the crural diaphragm during contraction and relaxation was measured on the fluoroscopic images. Five sequences of secondary peristalsis from each animal were analyzed. For these measurements, movement of the electrodes implanted in the crural diaphragm muscle was measured in reference to a nonmoving structure, i.e., a vertebral body. Fluoroscopic images were digitized every 1 s using an image grabber (Bravado 1000, Truvision, Indianapolis, IN), and distances were measured along the vertical axis using computer software (SigmaScan Pro,Jandel Scientific, San Rafael, CA). The data were compared using t-test and χ² test. Data are shown as means ± SE.

RESULTS

Effect of crural diaphragm stimulation on EGJ pressure. The EGJ pressure tracing in the absence of stimulation showed an end-expiratory pressure and an increase in the pressure with each inspiration. Electrical stimulation of the crural diaphragm, using electrodes implanted in the muscle, resulted in a sustained increase in the end-expiratory EGJ pressure (Figs. 2 and 3). The most reproducible responses were obtained when one pin from each electrode pair was used for stimulation. The increase in the EGJ pressure in response to electrical stimulation occurred rapidly, and the fall with the cessation of the stimulus was instantaneous.
neous (Fig. 2). The increase in EGJ pressure was directly proportional to the intensity of the electrical stimulus (Figs. 2 and 3). In two cats, the increase in EGJ pressure was \( > 40 \text{ mmHg} \) with the stimulus intensity of 10 mA. In the remainder, the maximal increase was 10–20 mmHg. The increase in EGJ pressure lasted as long as the electrical stimulation lasted; we tested up to 30-s-duration stimuli. Bilateral phrenicotomy abolished the spontaneous contractions of the costal and crural diaphragm. Electrical stimulation of the phrenic nerves, similar to muscle-implanted electrodes, resulted in an increase in EGJ pressure. During the period of stimulation there was a small increase in gastric pressure, most likely caused by the descent of the diaphragm. Pancuronium (at a dose of 0.2 mg/kg iv) blocked the EGJ pressure increase caused by phrenic nerve stimulation as well as the increase observed during the electrical stimulus delivered through the muscle-implanted electrode. (Fig. 4).

**Effect of esophageal balloon distension on EGJ relaxation.** In the absence of electrical stimulus to the crural diaphragm, esophageal distension induced relaxation of both components of the EGJ pressure, i.e., end-expiratory and inspiratory pressure. The EGJ relaxation occurred within 2–5 s after the onset of esophageal distension and lasted 5 s or longer after the balloon was deflated. Deflation of the balloon was usually followed by a secondary peristaltic contraction in the esophagus. The amplitude of EGJ relaxation was directly related to the balloon volume. The balloon volume required to induce a complete EGJ relaxation (>80%) in the absence of crural diaphragm stimulus was determined, and this balloon volume was then used for all subsequent esophageal distensions during crural diaphragm stimulation. Esophageal distension during the periods of increase in EGJ pressure induced...
by electrical stimulus to the crural diaphragm resulted in complete EGJ relaxation (Fig. 5). The completeness of EGJ relaxation appeared to be related to the occurrence of esophageal peristalsis. In other words, in the presence of secondary esophageal peristalsis there was complete relaxation of both components of the EGJ pressure, i.e., LES and crural diaphragm. The relaxation of the EGJ was nearly complete at all three electrical stimulus intensities studied, irrespective of the amplitude of the EGJ pressure increase induced by the electrical stimulus (Fig. 6).

Effect of esophageal peristalsis induced by a bolus on EGJ relaxation. Injection of 10 ml of water or barium induced a secondary esophageal peristaltic wave, which occurred 2–10 s after the injection. This contraction usually traversed the entire length of the esophagus. During this peristaltic contraction there was EGJ relaxation in the control period as well as during crural diaphragm stimulation periods (Figs. 6 and 7). In other words, in response to esophageal peristalsis, there was relaxation of both components of the EGJ, i.e., LES and crural diaphragm. The EGJ relaxation was complete (>80%) during control as well as crural diaphragm stimulation with 5-, 10-, and 15-mA electrical stimuli. There was no significant difference in percent EGJ relaxation between the control and electrical stimulation periods (Fig. 7).

Motion of crural diaphragm and esophageal transit of barium bolus during crural diaphragm stimulation. The motion of the crural diaphragm was studied on fluoroscopic images by monitoring the movement of the electrodes anchored to the crural diaphragm (Fig. 8) in reference to a fixed structure, i.e., vertebral body. Electrical stimulation of the crural diaphragm resulted in an aboral movement of 18.4 ± 0.8 mm that coincided with the increase in EGJ pressure. The electrodes remained in the descended position for the duration of the electrical stimulus, and cessation of the stimulus resulted in an orad movement of the electrodes. The latter coincided with the fall in EGJ pressure. During the periods of stimulation, a bolus-induced peristalsis resulted in an orad movement of the crural diaphragm.
However, the reversal of crural diaphragm movement during peristalsis was partial (3.8 ± 0.5 mm). At a time when the EGJ pressure recorded complete relaxation, the crural diaphragm moved only 20.2% (4.9–48.7%) of the maximal aboral movement induced by electrical stimulation. The fluoroscopic images revealed complete emptying of the esophagus in the control as well as during electrical stimulation to crural diaphragm.

**Phrenic nerve stimulation and EGJ relaxation.** Bilateral phrenic nerve stimulation increased EGJ pressure from 6.04 ± 0.58 to 25.11 ± 1.58 mmHg (P < 0.0001). Esophageal distension during stimulation of the phrenic nerves induced nearly complete EGJ relaxation (74.89 ± 10.70%). Bilateral vagotomy did not change the esophageal distension-induced EGJ relaxation (Fig. 9). Esophageal peristalsis-induced EGJ relaxation in this acute cat model could not be studied because neither balloon distension nor bolus injection induced esophageal peristalsis.

**DISCUSSION**

Our studies show that electrical stimulation of the crural diaphragm, either through electrodes implanted in the muscle or through the phrenic nerve, increases the EGJ pressure. The increase in EGJ pressure caused by electrical stimulus is directly proportional to the intensity of the electrical stimulus and lasts for the duration of the stimulus. Pancuronium blocks the increase in EGJ pressure induced by electrical stimu-
luses applied through electrodes implanted in the muscle and applied to the phrenic nerve. Because pancuronium is a competitive antagonist of the nicotinic cholinergic receptors at the skeletal muscle neuromuscular junction, our findings would indicate that the muscle contraction induced by electrical stimulation through phrenic nerve and muscle-implanted electrodes is mediated through stimulation of the neuromuscular junction rather than direct muscle excitation.

The LES muscle tone is largely caused by myogenic and neurogenic influences. Esophageal distension and peristalsis are stimuli that cause LES relaxation (10). The LES relaxation induced by esophageal distension can be mediated through either a central or peripheral mechanism. The central mechanism consists of vagal afferents, brain stem, and vagal efferents. The peripheral mechanism consists of myenteric plexus. Esophageal distension causes LES relaxation in the bilateral vagotomized animals through stimulation of the peripheral mechanism via myenteric neurons and release of inhibitory neurotransmitter (10). Nitric oxide appears to be the major inhibitory transmitter because nitric oxide antagonists abolish LES relaxation induced by a swallow and vagal nerve stimulus (9).

The increase in EGJ pressure during spontaneous inspiration is caused by crural diaphragm contraction. These spontaneous inspirations are the result of rhythmic activity of inspiratory brain stem neurons. Our study shows that, similar to spontaneous inspirations, an electrical stimulus delivered through phrenic nerves or electrodes implanted in the muscle can induce crural diaphragmatic contraction and an increase in EGJ pressure. Our study shows that esophageal distension and peristalsis can induce relaxation of the EGJ during the periods of crural diaphragm stimulation, when EGJ pressure is the result of contraction of both LES and crural diaphragm muscle. Because crural diaphragm contraction in our experiments was induced by stimulation of the neuromuscular junction, its relaxation during esophageal distension and peristalsis could only be the result of inhibition at the level of either the neuromuscular junction or the muscle.

A phenomenon similar to the one that we observed, i.e., a peripheral mechanism of inhibition in a skeletal muscle, was observed but interpreted differently by Asoh et al. (4). These investigators stimulated upper esophageal sphincter (cricopharyngeus muscle) contraction by a direct electrical stimulus and observed that a concurrent contraction of the myhyoid muscle induced relaxation of the sphincter. Their interpretation was that longitudinal pull exerted by myohyoid and other strap muscles on the upper esophageal sphincter causes its forced opening.

Could the crural diaphragm relaxation that we recorded, by measuring changes in EGJ pressure, be a mechanical artifact rather than a true relaxation? Other investigators have used EGJ pressure and or crural diaphragm EMG (1, 20, 23, 31) to study crural diaphragm inhibition. In our experiments, we could not measure the crural diaphragm EMG because the electrical pulses that we delivered to stimulate the neuromuscular junction induced a large artifact in our recordings. However, it is highly unlikely that the EGJ relaxation that we recorded using the manometric method is an artifact because 1) a number of investigators have used pressure recording as a standard method of measuring LES relaxation (4, 10, 21); 2) there is an excellent correlation between the increase in crural diaphragm EMG and EGJ pressure during diaphragmatic contraction and relaxation (1, 6, 22); 3) we observed that the EGJ was wide open on the X-ray images during transit of a bolus induced by esophageal peristalsis; and 4) fluoroscopic images showed that the crural diaphragm moved in the aboral direction in response to electrical stimulation (contraction) and in the reverse direction during peristalsis (relaxation). We believe that the aboral and orad movements of the crural diaphragm during electrical stimulation and peristalsis are evidence of crural diaphragm muscle contraction and relaxation, respectively. The orad movement during peristalsis in our experiments was incomplete in the presence of complete EGJ relaxation, the reason for which is not clear. The crural diaphragm has two types of muscle fibers; the inner ones are oriented in a circular fashion, and the outer ones are arranged in a more vertical fashion. It is very likely that contraction of the inner fibers leads to an increase in the EGJ pressure and contraction of the outer fibers leads to the vertical movement of the diaphragm, which would be important for the ventilatory function of the diaphragm. One possibility is that esophageal distension causes inhibition of the inner fibers, the ones responsible for sphincter function and not the outer one that causes vertical movement.

Under normal physiological conditions crural diaphragm muscle contraction is the result of oscillatory activity of the brain stem inspiratory neurons (11, 29). Even though direct proof is lacking, the available information points towards a central mechanism of inhibition of the crural diaphragm (3, 8, 27). The major reason is that unlike gastrointestinal smooth muscle, in which an inhibition can occur at the level of smooth muscle through the release of inhibitory neurotransmitter from the myenteric plexus, the skeletal muscle is not considered to have inhibitory innervation. Excitation and inhibition in a skeletal muscle are thought to occur because of inhibition of the activity of nerve cells located in either the central nervous system or the spinal cord. However, recent evidence indicates that there may be a mechanism of inhibition at the level of the skeletal muscle neuromuscular junction. Immunohistochemical staining shows the presence of neuronal nitric oxide synthase in the skeletal muscle (15, 24). The force-frequency (electrical stimulus) curves of costal diaphragm are shifted to the right by nitric oxide, suggesting that, similar to smooth muscle, nitric oxide can cause relaxation in a skeletal muscle (15). Additional evidence that there may be an inhibitory innervation at the neuromuscular junction comes from the immunohistochemical staining studies of the rat esophagus (25). These studies indicate that the NADPH diaphorase (a maker of nitric oxide)-containing nerve...
fibers from the myenteric neurons innervate the skeletal muscle end plate of the rat esophagus. It is possible that nitric oxide is an inhibitory neurotransmitter at the skeletal neuromuscular junction. Indeed, nitric oxide has been shown to block the neurosynaptic transmission across the skeletal muscle neuromuscular junction (32). Our preliminary studies show that sodium nitroprusside (a donor of nitric oxide) can block the release of acetylcholine from the neuromuscular junction (16).

The inhibition of the crural diaphragm in response to esophageal distension was not abolished by bilateral phrenicotomy and bilateral vagotomy. These observations would indicate that CNS processing of the esophageal afferents is not required for crural diaphragm inhibition, an observation similar to the one made by Miller et al. (19) in association with vomiting. On the other hand, a number of investigators have observed that bilateral vagotomy abolishes the esophagocutaneous diaphragmatic inhibitory reflex (1, 8, 27), an observation contrary to ours. Our experimental design was unique in that we studied inhibition of crural diaphragm muscle that was contracted by direct stimulation of the neuromuscular junction. Other investigators studied inhibition of the spontaneous crural diaphragm contraction, which is mediated through the brain stem neurons. Ours is the first study that shows evidence for a mechanism of inhibition at the level of neuromuscular junction in crural diaphragm. However, our data only identify the presence of a peripheral inhibitory mechanism; they do not refute the possibility of a central mechanism of inhibition for crural diaphragm contractions. Further studies are needed to determine the extent of central and peripheral contributions to the inhibition of spontaneous crural diaphragm contractions. Further studies are also required to investigate the nature of the peripheral inhibitory mechanism, i.e., the peripheral neural pathway between the esophagus and crural diaphragm and the noninhibitory transmitter.

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