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Research Article

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Lower Esophageal Sphincter Relaxation: Studies on the Neurogenic Inhibitory Mechanism

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ABSTRACT The purpose of this study was to determine the physiological mechanism of lower esophageal sphincter (LES) relaxation. Circular muscle of the esophagus, LES, and stomach were evaluated for their inhibitory response to electrical stimulation during a maintained tonic contraction produced by a superfusion of acetylcholine and physostigmine. Only the circular muscle of the distal esophagus showed an inhibitory response to electrical stimulation. The maximal inhibition of LES muscle was 63.9±5.9 (mean±SE) % of the acetylcholine produced tension and occurred at 80 V. Upper esophageal and gastric muscle were not inhibited. The inhibitory response of the LES muscle was antagonized by tetrodotoxin and hexamethonium but not by other specific antagonists. Adrenergic nerve destruction following 6-hydroxydopamine also did not abolish the LES inhibition. These data indicate that the distal esophagus, at the zone of the manometrically determined LES, is characterized by a nonadrenergic neural inhibitory system. We suggest that these nerves may mediate LES relaxation.

INTRODUCTION

The uninterrupted progression of a bolus from esophagus to stomach is dependent upon the integrated functions of esophageal peristalsis and lower esophageal sphincter (LES)[1] relaxation (1–3). When either one or both of these functions are impaired, esophageal transport may be altered. In patients with achalasia, the impaired relaxation of the LES during swallowing is a major determinant of the clinical manifestations of this disease (1). Despite our knowledge of the behavior of the LES in man, little is known of the basic mechanism of LES relaxation. The purpose of this study is to utilize LES and adjacent circular smooth muscle in vitro, to determine the presence of an inhibitory mechanism that might account for LES relaxation in vivo, and to evaluate the physiologic basis of this inhibitory system.

METHODS

33 adult opossums (*Didelphis virginiana*) of both sexes, weighing 2.4–5.5 kg were studied. Each animal was anesthetized with 40 mg/kg of intraperitoneal pentobarbital and strapped supine to an animal board for studies in vivo. Intraluminal manometry was performed with three open-tipped catheters, constantly infused with distilled water at 1.2 ml/min. Pressures were transmitted to Statham transducers (P23BB) with its signal directed to a direct writing Beckman recorder. The lower esophageal sphincter was identified by manometry and the recording apparatus was secured to the lower jaw with the second orifice within the LES (4). The animals were then killed by intravenous pentobarbital and the chest and abdomen were opened. The orifice within the LES was identified and a second tube, which served as a marker for the LES, was passed by mouth and positioned at the LES, as previously determined by manometry. The recording tube was then withdrawn. The esophagus was ligated around the second tube at a point 8–10 cm proximal to the anatomic gastroesophageal junction where the narrow esophagus flares into the stomach. The pylorus was ligated distally. The entire esophagus and stomach were transferred to organ baths of Krebs-Ringer solution (Krebs-Ringer solution composition (mM): Na^+ 138.6, K^+ 4.6, Ca^{++} 2.5, Mg^{++} 1.2, Cl^- 126.2, HCO_3^- 21.9, PO_4 1.2, glucose 49.6), bubbled with 95% O_2 and 5% CO_2 at 37°–38°C. The esophagus was separated from the stomach at the anatomic gastroesophageal junction. The serosal tissues were cleaned and the mucosa was removed by sharp dissection. The LES, as determined from manometry, was 0.5–1.0 cm above the anatomic gastroesophageal junction and was approximately 1.5 cm in length. The LES and successive levels above it and below it, the gastric fundus and

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[1] *Abbreviations used in this paper:* Ach, acetylcholine; C_6, hexamethonium; LES, lower esophageal sphincter; L_o, length of optimal tension development; TTX, tetrodotoxin.

antrum were cut into smooth muscle strips 0.5 cm in width and 1.0 cm in length. The muscle was oriented to record tension from the plane of contraction of the circular muscle.

In Fig. 1 is shown a diagram of the superfused system in which the muscle strips were studied. Six muscle strips were studied simultaneously. The muscles were superfused with a Krebs-Ringer solution bubbled with 95% O_2 and 5% CO_2 at 35–38°C. The strips were mounted in the appropriate plane to record the isometric tension of the circular muscle. Force transducers (Grass Ft. 03C) measured isometric tension which was graphed on a direct writing Beckman recorder. The length of optimal tension development, L_o, was determined for all strips as previously described (4). The muscles were then set at their respective L_o for the remainder of the experiment. Two platinum wire stimulating electrodes imbedded in movable lucite arms were adjusted to juxtapose the lateral longitudinal muscle strip surfaces. A square wave stimulator (Grass stimulator model S44, with stimulus isolation unit SIU 5) delivered 30 s trains of 1 ms duration direct current square wave pulses at 10 cycles/s. The voltage was varied from 10 to 100 V. The frequency and pulse duration were selected from preliminary studies. Both parameters gave consistent high amplitude contractions at the termination of electrical stimulation. Pulses of lower duration did not consistently elicit contractions. Pulses of longer duration irreversibly damaged the muscle. Stimulus frequency was varied in several studies as noted below. The pulse strength in the text are those delivered from the stimuli source. Pulse strength at the electrodes was reduced by 20%.

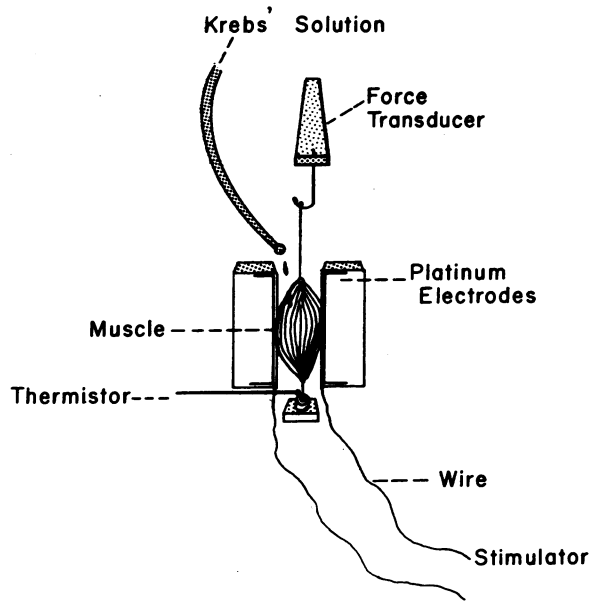

FIGURE 1 Diagrammatic illustration of the apparatus used to study the response of a single muscle strip. Heated and oxygenated Krebs' solution was superfused from a reservoir above the muscle. All compounds were added to the reservoir. The muscle was secured to a fixed site distally and the transducer proximally. Electrical field stimulation was applied across platinum wires at either side of the muscle. A thermistor was used to monitor the temperature of the superfusate.

TABLE I
Drugs and Sites of Action

Name	Abbreviation	Action
Hexamethonium chloride	C_6	Ganglionic blocker
Tetrodotoxin	TTX	Selective blockade of axonal conduction by the prevention of sodium permeability
Phentolamine mesylate		Adrenergic (α)-blocker
Propranolol hydrochloride		Adrenergic (β)-blocker
Physostigmine sulfate		Anticholinesterase
Acetylcholine chloride	Ach	Parasympathomimetic
Methysergide bimaleate		Antagonism of serotonin on smooth muscle
6-Hydroxydopamine		Destruction of adrenergic nerve terminals

Initially, each muscle was evaluated for the response to electrical stimulation during superfusion of Krebs-Ringer solution. Following this initial evaluation, the muscles were intermittently superfused with acetylcholine (Ach) and physostigmine to establish sustained plateaus of contraction. Upon this sustained contraction, each muscle was tested for its response to electrical stimulation at different voltages. The tension recorded prior to stimulation and the nadir of tension during stimulation were used to calculate percent inhibition.

In muscles that showed inhibition with electrical stimulation, antagonists were evaluated to determine the mechanism of this effect. Each antagonist was added to the superfusate along with the Ach and physostigmine and allowed to perfuse the muscle for several minutes prior to electrical stimulation. The antagonists were used at a molar concentration that gave near maximal inhibition of the peak response to its respective agonist. At least 15 min were allowed between the testing of any antagonist. In addition, four animals were given 6-hydroxydopamine (100 mg/kg, intraperitoneal) 24 h prior to study. Muscle from these animals were studied in an identical manner. All drugs, abbreviations, and sites of action are summarized in Table I.

RESULTS

In Fig. 2 is shown the response of non-Ach perfused LES circular muscle to electrical stimulation. At the onset of stimulation, a brief, low amplitude contraction was elicited. This was followed by a quiescent period for

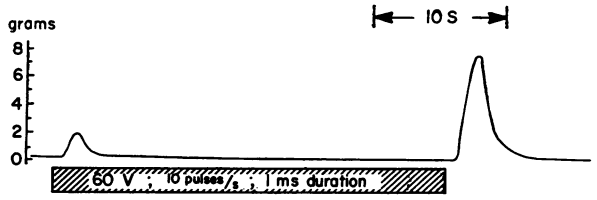

FIGURE 2 The response of non-acetylcholine perfused lower esophageal sphincter circular muscle to electrical stimulation. The muscle which was not spontaneously active showed a low amplitude, brief contraction with the onset of stimulation and a higher amplitude contraction after a short latent period following the cessation of the stimulus. No change in muscle tension was recorded during the stimulus.

the duration of the stimulus. Upon cessation of the stimulus, a high amplitude contraction followed a brief latent period. This type of response was elicited in all esophageal and LES circular muscle studied. The low amplitude contraction at the onset of stimulation was recorded consistently only during the initial portion of the study. The other characteristics were consistent throughout the study period.

In Fig. 3 is shown the responses of tonically contracted LES, esophageal, and gastric circular muscle to electrical stimulation. Each muscle was contracted by a superfusate of Ach and physostigmine. Only the LES muscle showed a reduction in active tension. At the break in the electrical stimulus, the LES and esophageal muscles showed a prominent contraction and then returned to their previous active tensions. The gastric muscle showed neither inhibition with electrical stimulation nor the prominent contraction at the break in stimulation. This response was identical for fundal and antral circular muscle.

In Fig. 4 is shown the stimulus response characteristics of all muscles studied. LES muscle showed a good

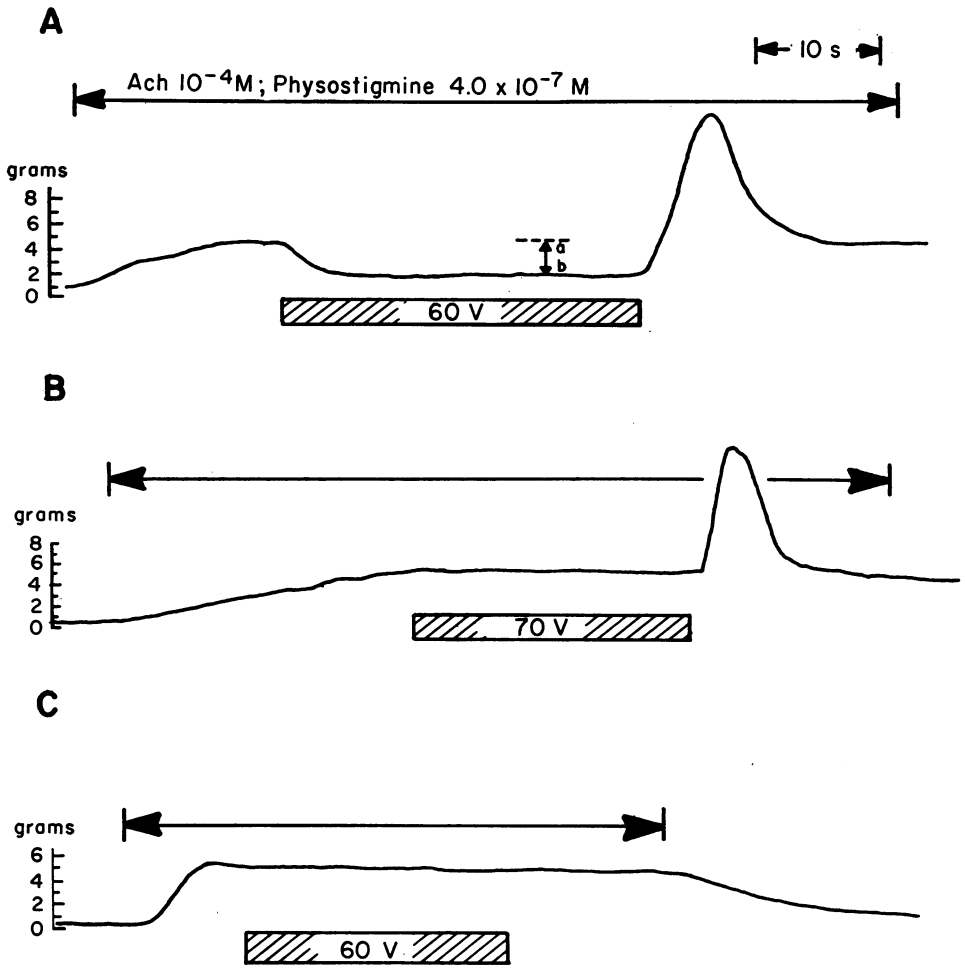

FIGURE 3 The response of electrical stimulation upon acetylcholine contracted LES, esophageal, and gastric circular muscle. Each muscle was superfused with acetylcholine (10^{-4} M) and physostigmine (4.0×10^{-7} M) as indicated on each tracing. All electrical stimulation was administered at 10 pulses/s, 1 ms duration. (*A*) LES muscle: At the onset of electrical stimulation, the muscle was inhibited. This inhibition remained for the duration of the stimulus. At the cessation of the stimulus, the muscle showed a prominent contraction and then returned to the tension achieved prior to stimulation. The inhibitory response was calculated from points *a* to *b*. (*B*) Esophageal muscle (4 cm above the LES): No inhibition was recorded during stimulation but the prominent contraction at the cessation of stimulation was present. (*C*) Gastric muscle: No inhibition was recorded during stimulation and no contraction was seen at the termination of the stimulus.

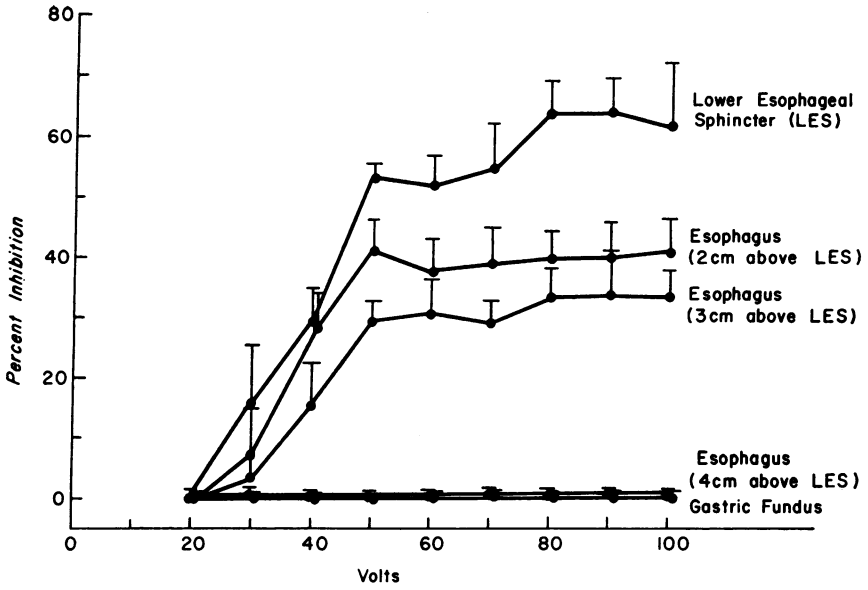

FIGURE 4 The stimulus response characteristics of circular smooth muscle from esophagus and stomach. The inhibitory response of acetylcholine contracted muscle is plotted as a function of increasing voltage. Each point represents the mean plus one standard error of the mean for studies obtained on muscle strips from a minimum of eight animals.

relationship between stimulus and percent reduction in tension. The maximum reduction in tension occurred at 80 V and represented 63.9±5.9 (mean ±SE) percent decrease in tension. Esophageal circular muscle from 2.0 cm and 3.0 cm. above the LES showed stimulus related decreases in tension. The absolute magnitude of this inhibition was less than that recorded from the LES circular muscle. Esophageal circular smooth muscle, 4.0 cm above the LES, did not show significant inhibition at any voltage. Similarly, gastric muscle was not inhibited. Esophageal muscle (4 cm above the LES) and gastric muscle did not show an inhibitory response when stimulated at frequencies of 20, 30, 40, and 50 cycles/s at 20, 40, 60, and 80 V.

The mechanism of electrical inhibition of LES and esophageal circular muscle was investigated next. In Fig. 5 is shown the effect of different antagonists upon LES circular muscle inhibition. The data is graphed as a percent of control inhibitory response prior to addition of each antagonist. Tetrodotoxin, a specific neural antagonist (5, 6), markedly diminished electrical inhibition of LES circular muscle ($P < 0.001$). Hexamethonium produced a minimal but significant reduction in the inhibition in response to electrical stimulation ($P < 0.05$). Other specific antagonists and antagonist combinations did not significantly alter the magnitude of inhibition in response to electrical stimulation. Each antagonist was used at a concentration that would markedly reduce its appropriate maximum agonist response (7). These antagonists did not alter the response to Ach. These data indicated that electrical inhibition of this muscle was mediated through nerves and that these nerves did not release catecholamines or serotonin.

The nonadrenergic character of electric inhibition was confirmed in animals pretreated with 6-hydroxydopamine. This chemical, when administered 24 h prior to killing of the animal, destroyed the adrenergic neural system (8).[3] In Fig. 6 is shown a comparison of LES circular muscle inhibition in control animals and in four animals pretreated with 6-hydroxydopamine. Electrical inhibition was identical, confirming that this response was mediated by nonadrenergic nerves.

DISCUSSION

These data indicate that the distal esophagus of the opossum, including the zone of the manometrically defined LES, possesses a neurogenic inhibitory system that is nonadrenergic. It is suggested that these nonadrenergic nerves may mediate the response of LES relaxation during swallowing.

To demonstrate inhibition in an isometric recording of muscle, the muscle must either be spontaneously active or it must be contracted prior to its inhibition (6,

[3] Specimens of esophagus and stomach from four treated and four control animals were evaluated by Dr. David Jacobowitz of the National Institutes of Health. Both histochemical staining and direct assay for norepinephrine demonstrated findings consistent with denervation in animals pretreated with 6-hydroxydopamine.

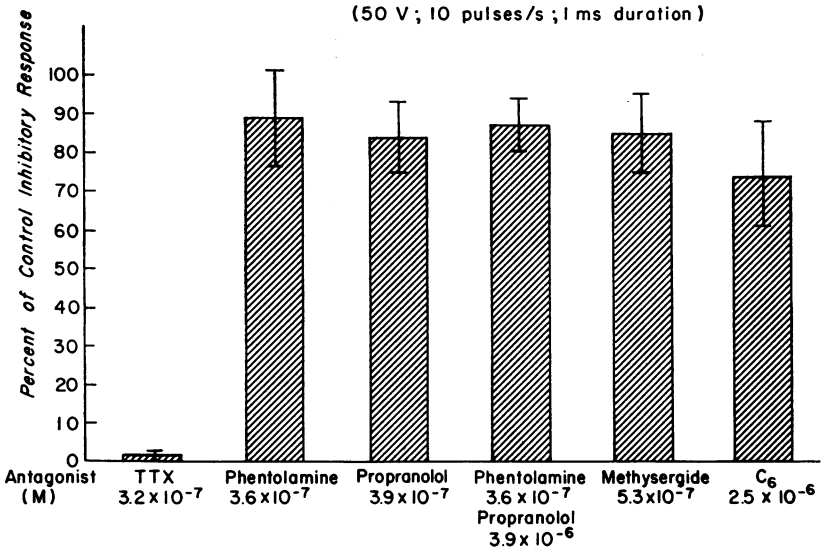

FIGURE 5 The effect of antagonists on the inhibitory response to 50 V on the lower esophageal sphincter circular muscle. All data is expressed as a percent of the control response. Tetrodotoxin (TTX) markedly diminished the inhibitory response ($P < 0.001$) and hexamethonium (C_6) produced a slight reduction in this response ($P < 0.05$). Each bar represents the data obtained from muscle strips taken from a minimum of eight animals.

9). Because esophageal muscle was not spontaneously active, it was first contracted by acetylcholine and physostigmine. This drug combination was selected for two reasons. First, the resting pressure of the opossum LES occurs mainly through the action of gastrin which in turn acts through acetylcholine release (7, 10). Second, only by combining physostigmine with acetylcholine could we maintain a level plateau of contraction. The tonic contraction elicited by these compounds was used to evaluate the inhibitory response to electrical stimulation.

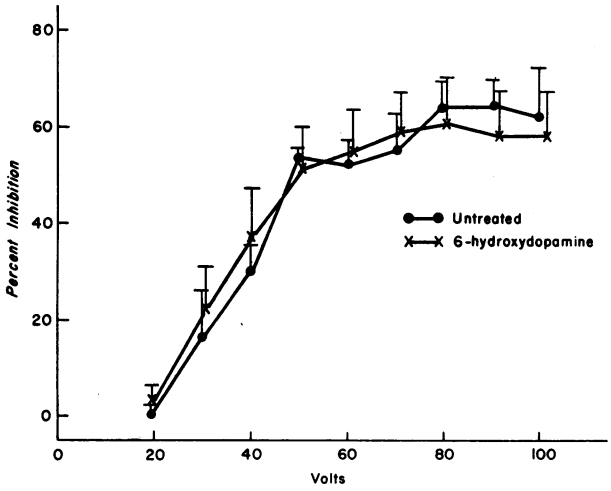

FIGURE 6 The stimulus response characteristics of lower esophageal sphincter circular muscle from 12 control animals and four animals pretreated with 6-hydroxydopamine.

Three types of response were obtained during electrical stimulation of tonically contracted muscle. The LES muscle showed a prominent phase of inhibition which began promptly upon stimulation and which corresponded to the quiescent phase of electrical stimulation of noncontracted LES muscle (Fig. 1). The cessation of stimulation yielded the prominent contraction similar to that seen in non-acetylcholine perfused LES muscle. Muscle from just above the LES gave a similar response but with less inhibition at higher voltages. The second type of response was recorded in muscle from the upper esophagus, 4 cm above the LES. Inhibition was not recorded at any voltage or stimulus frequency but the prominent contraction at the cessation of the stimulus was present. The third type of response was demonstrated in gastric muscle. This muscle showed no inhibition with electrical stimuli and no contraction at its termination. Thus, only esophageal circular muscle demonstrated the prominent contraction at the cessation of stimulation and only the distal esophageal muscle was inhibited during electrical stimulation.

The response to electrical stimulation was evaluated further by quantifying LES muscle inhibition in the presence of specific antagonists. LES muscle inhibition was abolished by tetrodotoxin, a neural antagonist (5, 6). Other selective antagonists, in concentrations adequate to abolish their respective maximum agonist re-

sponse, did not alter LES muscle inhibition (7). Likewise, muscle from animals adrenergically denervated with 6-hydroxydopamine still showed an inhibitory response to electrical stimulation (8). Thus, the inhibition recorded in LES muscle was mediated through nonadrenergic nerves.

The nonadrenergic neural inhibitory system recently has received considerable attention (9, 11–13). It has been suggested that these nerves release either adenosine triphosphate or a related purine compound (11). The presence of nonadrenergic inhibitory nerves was demonstrated in spontaneously active muscle in which the activity was diminished by electrical stimulation (9, 11). The inhibitory effect of nonadrenergic nerves is through its effect upon muscle membrane potential. These nerves hyperpolarize muscle producing diminished excitability (12). The prominent contraction at the termination of the electrical stimulation was described in muscles with nonadrenergic inhibitory nerves. This response, called either rebound excitation (12) or an "off" response (13, 14), was due to muscle depolarization following the rapid return of hyperpolarized muscle to resting potential.

Based on these previous publications dealing with nonadrenergic inhibitory nerves, one might predict that all esophageal muscle should be inhibited during electrical stimulation since all esophageal muscle had an "off" response. However, in this study only the distal esophageal circular muscle showed inhibition. This discrepancy is not readily explained. Several possible explanations can be given. First, the experimental design selectively allowed us to elicit an inhibitory response in one muscle and not in the other. Second, electrical stimulation of the upper esophageal muscle elicited a response from excitatory nerves which masked the inhibitory response. Third, inhibition and rebound excitation or "off" response are distinct responses, both mediated through nonadrenergic, noncholinergic nerves. Under the experimental conditions of this study, inhibitory nerual function could only be shown at the distal esophagus and LES. The presence of inhibitory nerves in the upper esophagus cannot be excluded. Their presence may be established by other experimental techniques.

It is possible that human LES relaxation is also mediated through nonadrenergic inhibitory nerves. The human LES is similar in structure and physiological behavior to the opossum LES (4, 7, 13, 14). The LES is a portion of the esophagus which is characterized by an elevated intraluminal pressure that is regulated through both neural and humoral factors. The LES responds to changes in intra-abdominal pressure through a cholinergic neural mechanism (15) and is modified by the endogenous release of the hormones, gastrin and secretin (16). Despite these mechanisms to alter the level of sphincter pressure, the response to swallowing is the same. Upon swallowing, LES pressure falls to a nadir which approximates the level of intra-abdominal pressure (1). When peristalsis has traversed the esophagus, the LES briefly contracts to a pressure beyond the resting level. An attractive hypothesis is that LES relaxation and contraction in vivo correspond to inhibition and rebound excitation, respectively, as demonstrated here in vitro. Support for the hypothesis that human LES relaxation and contratcion are mediated through a nonadrenergic neural inhibitory system is limited. Studies designed to pharmacologically antagonize the LES response to swallowing have not been specifically performed. However, limited observations indicate that cholinergic (17) and beta adrenergic antagonists (18) do not alter LES relaxation in man. Human LES circular muscle has not been evaluated in response to electrical stimulation. Although these studies on opossum LES muscle must be interpreted conservatively and not directly applied to man, they may serve as a guide to further investigation of the mechanism of sphincter relaxation in normal man and in patients with achalasia.

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