Feline lower esophageal sphincter sling and circular muscles have different functional inhibitory neuronal responses

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L’Heureux, Marie-Claude, Ahmad Muinuddin, Herbert Y. Gaisano, and Nicholas E. Diamant. Feline lower esophageal sphincter sling and circular muscles have different functional inhibitory neuronal responses. Am J Physiol Gastrointest Liver Physiol 290: G23–G29, 2006. First published September 15, 2005; doi:10.1152/ajpgi.00303.2005.—The lower esophageal sphincter (LES) has a circular muscle component exhibiting spontaneous tone that is relaxed by nitric oxide (NO) and a low-tone sling muscle that contracts vigorously to cholinergic stimulation but with little or no evidence of NO responsiveness. This study dissected the responses of the sling muscle to nitric innervation in relationship to its cholinergic innervation and circular muscle responses. Motor responses were induced by electrical field stimulation (EFS; 1–50 Hz) of muscle strips from sling and circular regions of the feline LES in the presence of cholinergic receptor inhibition (atropine) or NO synthase inhibition [Nω-nitro-L-arginine (L-NNA) ± atropine]. This study showed the following. First, sling muscle developed less intrinsic resting tone compared with circular muscle. Second, with EFS, sling muscle contracted (most at ±10 Hz), whereas circular muscle relaxed >50% by 5 Hz. Third, on neural blockade with atropine or L-NNA ± atropine, 1) sling muscle, although predominantly influenced by excitatory cholinergic stimulation, had a small neural NO-mediated inhibition, with no significant non-NO-mediated inhibition and 2) circular muscle, although little affected by cholinergic influence, underwent relaxation predominantly by neural release of NO and some non-NO inhibitory influence (at higher EFS frequency). Fourth, the sling, precontracted with bethanecol, could relax with NO and some non-NO inhibition. Finally, the tension range of both muscles is similar. In conclusion, sling muscle has limited NO-mediated inhibition to potentially augment or replace sling relaxation effected by switching off its cholinergic excitation. Differences within the LES sling and circular muscles could provide new directions for therapy of LES disorders.

MATERIALS AND METHODS

Muscle strip studies. All experimental procedures were approved by the University Health Network Animal Care Committee. Fasted, adult cats of either sex, weighing 2.5–5.0 kg, were anesthetized with ketamine hydrochloride (0.15 ml/kg im, Bimeda-MTC; Cambridge, Ontario, Canada) and euthanized with pentobarbital sodium (0.5 ml/kg iv, Bimeda-MTC). At laparotomy, an esophagogastrectomy segment from 5 cm above the LES and including a 4-cm cuff of the stomach was carefully excised and placed into Krebs solution equilibrated with 95% O2-5% CO2 and maintained at pH 7.40 ± 0.05. The tissue was freed from the surrounding fascia, stretched to its in situ length, and then cut along the greater curvature of the stomach. The mucosa was then gently removed to expose the LES circular and sling regions (38). Muscle strips, 2 mm wide and 8 mm long, were obtained from the long axis of the circular and oblique sling muscles. Muscle strips were individually mounted in a 25-ml water-jacketed tissue bath. One end was secured to an electrode holder, and the other end was fastened to an isometric force transducer (model FT-03, Grass Instruments; Quincy, MA) supported on a rack and pinion clamp (Harvard Apparatus; Holliston, MA). Output data from the force transducer were acquired using a DigiData 1200B analog-to-digital converter (Axon Instruments, Foster City, CA) and a Digidata 1222 analog-to-digital converter (Axon Instruments). Tension recordings were made on a Macintosh computer and analyzed using pCLAMP (Axon Instruments). Tissues were allowed to equilibrate for 1 h and were then stimulated isometrically at 10 Hz with 100 μA (EFS, 0.1 ms). In some cases, EFS was varied frequencies with particular attention to the neural NO-mediated inhibitory effects. The findings of NO-mediated effects were assessed in relation to the interaction with the accompanying cholinergic excitatory effects and compared with the responses in the LES circular muscle. We hypothesized that the NO-mediated effects would be less in the sling muscle. A portion of this work has appeared in abstract form (21).

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Instruments; Union City, CA). pCLAMP software (version 8, Axon Instruments) was used to visualize and analyze data. Transmural EFS (0.5-ms square-wave pulses; 70 V; 5-s train; frequencies of 1, 2, 3, 5, 10, 20, and 30 Hz) was delivered by a Grass stimulator (SP-9) through platinum wire electrodes on either side of each muscle strip.

Before the experiment, muscle strips were hung with 0.5-g tension for a 1-h equilibration period, and the length was then measured and defined as the initial length (L₀). Strips were then slowly stretched twice at increments of 25% of L₀ with 15 min between each stretch (30). At a study length of 150% L₀, EFS of the sling muscle resulted in an initial contraction in all strips and relaxation of the circular muscle (6). Motor responses to EFS were sequentially assessed over a frequency range of 1–30 Hz with 5 min between each frequency under control conditions (no added drugs) and in the presence of 1 atropine (a cholinergic receptor inhibitor, 10⁻⁶ M) and 2) N⁵-nitro-L-arginine (L-NNA; an NOS inhibitor, 10⁻⁴ M) ± atropine (10⁻⁶ M).

The chemicals were allowed 15 min to act on the strips. To address whether or not a sling muscle can also exhibit nitricergic relaxation to EFS, studies were performed after tone of the sling had been increased by precontraction with thenalcaliform (a cholinergic agonist, 10⁻⁵ M (30)) ± L-NNA (10⁻⁴ M). Finally, to evaluate whether the two LES muscles have efficient responses to NO, the nerves were blocked with tetrodotoxin (TTX; 10⁻⁶ M), and a dose-response curve was performed with the NO donor sodium nitroprusside (SNP; from 10⁻⁸ to 10⁻³ M, with 5 min allotted between each dose). A 30-min incubation period was allowed after the addition of thenalcaliform, L-NNA, TTX, or ACh.

Chemicals. Atropine (atropine sulfate), L-NNA, TTX, ACh chloride, and SNP were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), and thenalcaliform (bethanechol chloride) was from Research Biochemicals (Natick, MA). Atropine, thenalcaliform, TTX, ACh, and SNP were dissolved into deionized distilled water. L-NNA (75 mg) was added into 5 ml of 0.1 N HCl and heated gently until dissolved, and the volume made up to 10 ml with water. Krebs solution (115 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO₄·7H₂O, 1.2 mM NaH₂PO₄·H₂O, 22 mM NaHCO₃, 2.0 mM CaCl₂·2H₂O, and 11 mM dextrose) was maintained at 37°C with 95% O₂-5% CO₂ in the organ bath.

Result analyses. Data were normalized and expressed as tension as follows: tension (mN/mm²) = [tone (g) x 9.81 m/s²]/[cross-sectional area (mm²)], where cross-sectional area (mm²) = [tissue weight (mg)]/[1.05 mm² x study length (mm)] and 1.05 mm² is the density of smooth muscle (4). When muscle strips were not stimulated before the addition of any drug, the tension was referred to as baseline tension. At the end of an experiment, each muscle strip was blotted onto a filter paper and weighed with an analytical balance. No statistical differences were found between the average weight of the LES sling versus circular muscle strips (0.052 ± 0.003 vs 0.044 ± 0.003 g, respectively; P > 0.05).

All data are expressed as means ± SE, where n represents the number of muscle strips studied (10–23) per group of 3–7 cats/ experiment. Each muscle strip served as its own control. SAS software (SAS Institute; Cary, NC) was used to determine statistical differences between groups, frequencies, and interaction between the former variables by a repeated-measures ANOVA, followed by a post hoc Bonferroni adjusted pair-wise comparison test. Student’s t-test was also used to determine statistical differences between means. An α-value of 0.05 was considered as significant.

RESULTS

Effect of atropine. As shown in Fig. 1, under control conditions, the sling muscle developed significantly less resting tension than the circular muscle (7.16 ± 1.40 vs 15.23 ± 1.60 mN/mm², P < 0.01). With EFS at increasing frequencies, the sling muscle contracted (Fig. 1A). The rate of contraction was greatest at ≤10 Hz (to 19.60 ± 3.83 mN/mm² at 10 Hz, P < 0.05). The rate became less pronounced at higher frequencies, with the contraction amplitude reaching 29.87 ± 5.90 mN/mm² at 30 Hz. In contrast, the circular muscle underwent progressive relaxation (Fig. 1B); relaxation was most rapid at lower frequencies (≤5 Hz), to >50% at 5 Hz (to 4.68 ± 0.47 mN/mm², P < 0.001), with little further relaxation at higher frequencies (3.94 ± 0.47 mN/mm² at 30 Hz).

Atropine was added to investigate the relative cholinergic neural influence. For the LES sling muscle (Fig. 1A), atropine resulted in an insignificant decrease of the resting tone (from 7.16 ± 1.40 to 6.56 ± 1.08 mN/mm²) and virtually abolished the contraction at all frequencies (P < 0.001). Therefore, in the sling muscle, there is a marked neural cholinergic excitatory influence but little or no neural inhibitory influence evident. For the LES circular muscle (Fig. 1B), the addition of atropine resulted in a small but insignificant increase in the resting tension (from 15.23 ± 1.60 to 17.16 ± 1.84 mN/mm²) and a slight shift to the right of the relaxation responses at the lower frequencies from 1 to 5 Hz (P < 0.05). A largely unaltered relaxation response suggests that there is little neural cholinergic influence on the circular muscle.

Effect of NO. The relative contribution of the neural NO-mediated and non-NO-mediated inhibition was assessed using L-NNA alone or with atropine. Under control conditions, as noted before (Fig. 1), the sling muscle developed less sustained intrinsic resting tone compared with circular muscle (Fig. 2). Again, upon EFS, the sling muscle contracted (contracting most rapidly at ≤10 Hz; Fig. 2A), whereas the circular muscle relaxed (relaxing most rapidly at ≤5 Hz; Fig. 2B).

In the sling muscle, L-NNA increased the amplitude of its contraction over that of controls at EFS ≥10 Hz (Fig. 2A), suggesting that NO released from the nerves can cause inhibition of the sling muscle at higher EFS frequencies. In the circular muscle, L-NNA abolished the EFS-induced relaxation (Fig. 2B). In fact, a small contraction for the circular muscle was observed (P < 0.001), at times followed by a delayed relaxation at higher EFS frequencies, which would suggest that a small cholinergic excitatory influence was unmasked by the inhibition of NO. L-NNA + atropine rendered the sling muscle virtually unresponsive to EFS (from basal tone at 7.13 ± 1.65 to 6.55 ± 1.36 mN/mm² at 30 Hz, P > 0.05), and the cholinergic influence on resting tone (7.13 ± 1.65 vs. 9.75 ± 1.78 mN/mm², P > 0.05) was absent. With EFS, there was no significant inhibition of the sling at any frequency (Fig. 2A). Therefore, another non-NO inhibitory mechanism was not apparent in this set of experiments. However, a significant relaxation of the circular muscle could still be observed at EFS ≥5 Hz (Fig. 2B), suggesting the involvement of a non-NO neural inhibitory pathway at higher EFS frequencies.

As previously noted, for the sling muscle, the addition of atropine abolished the EFS-induced contraction, and only a small relaxation response was seen (Fig. 1A). With the subsequent addition of L-NNA, the majority of this relaxation was also abolished (Fig. 2A); the small decrease seen at higher frequencies was not significant. Therefore, in the cat, a significant non-NO-mediated inhibitory neural influence is likely not involved in the relaxation response of the sling muscle and is seen only in the circular muscle.
NO-mediated inhibition of precontracted LES sling muscle. To assess the ability of the sling to relax due to release of NO, studies were performed after elevation of its resting tone. Precontraction of the sling muscle with bethanecol led to an increase in its basal resting tone (to 60.4 ± 8.8 mN/mm²; Fig. 3). Upon EFS, the sling muscle relaxed, with the relaxation most rapid at lower frequencies (10 Hz), decreasing by 50% at 10 Hz versus the basal resting muscle tone (to 29.7 ± 4.6 mN/mm², P < 0.01). The tone stabilized at higher frequencies, providing a relaxation profile that varied some from that of the circular muscle (Figs. 1B and 2B). The maximum decrease in tension required higher frequencies in the sling. The addition of L-NNA resulted in significantly higher tensions at all frequencies (P < 0.001; Fig. 3), with some relaxation at higher frequencies. Therefore, the sling muscle is responsive to the neural release of NO at EFS frequencies ≤10 Hz and may have a small non-NO inhibitory influence at higher frequencies. To determine whether the less-sensitive NO-mediated inhibitory response to EFS in the sling was due to a muscle property rather than a property of the innervation, the NO donor SNP was added to the precontracted LES muscles after neural blockade with TTX. The dose-response inhibition was identical in sling and circular muscles (P > 0.05; Fig. 4). Therefore, both muscles are equally responsive to NO.

Range of LES muscle tension. The tension range covered by both muscles is very similar when viewed after neural blockade, as is evident when Fig. 2, A and B, is superimposed. That is, at the upper end, the low resting tone of the sling muscle augmented by the large cholinergic effect, is similar to that of the sum of the large resting tone of the circular muscle augmented slightly by the cholinergic excitation. Similarly, at the lower end of the range, the circular muscle will relax to approximately the same tension as seen in the resting sling muscle.

DISCUSSION

The present study further characterizes the regional differences between the sling and circular muscle components of the LES in the cat. For the first time, we assessed the nitricergic neural influence within the sling muscle. As previously demonstrated, the sling muscle has a low intrinsic resting tone compared with the circular muscle (30, 37, 38, 47, 51). This difference sets the conditions whereby excitatory and inhibitory innervations interact to increase or decrease tone in the two muscles. The sling muscle 1) is highly responsive to excitatory neural cholinergic influence; 2) has a limited neural NO-mediated inhibition, which is most obvious at higher frequencies; 3) may have a small non-NO-mediated inhibition; and 4) if precontracted, can be induced to relax with NO. In contrast, the circular muscle 1) has little neural excitatory cholinergic influence on the already elevated intrinsic tone, 2) has a reduction of the high intrinsic tone predominantly...
through the release of NO, and 3) has a non-NO neural inhibitory influence becoming evident at EFS ≥5 Hz. Therefore, in vivo, it is most likely that the sling normally relaxes predominantly by switching off the cholinergic vagal excitatory influence but also has some capacity to relax in response to release of NO. In contrast, the circular muscle would relax in large part through inhibition of its high intrinsic tone by release of NO.

A recent study by González et al. (11) explored the different participating excitatory and inhibitory neural inputs to circularly oriented muscle strips of the human LES. Although these strips were taken mainly from the left lateral aspect of the LES, their behavior and relaxation of elevated tone were similar to the circular muscle of the human LES described by Preiksaitis et al. (39) and of the cat in our present study, and not of the sling muscle. Significant myogenic tone with relaxation on EFS has been the defining characteristic of the LES circular muscle in studies of other species as well (5, 6). We have not seen this behavior in carefully isolated sling muscle. It is likely therefore that the tissue studied by González et al. (11) was largely LES circular muscle, although the 40 of 75 strips from the upper end of the LES that exhibited an “on” relaxation followed by an “off” contraction may have included some esophageal body component. Our findings in the LES circular muscle indicate that this muscle in the cat is similar to that in the human.

Evidence is accumulating that the sling muscle also contributes to the overall asymmetrical manometric LES pressure profile at rest in vivo and therefore to the functional characteristics of the LES. In the pig, a mechanical model has demonstrated that the combined contribution of both sling and circular muscles was necessary for correlating with the in vivo LES manometric profiles in the human (19). Our findings indicate that the two muscles contribute to LES function on the basis of their different inherent properties and responsiveness to their excitatory and inhibitory innervation. Maintenance of resting LES tone in the left lateral aspect, the location of the sling, appears to be largely due to the responsiveness of the sling muscle to its cholinergic innervation, normally vagally driven. Atropine decreases the leftward resting LES pressure in both the human and cat, with little effect on the pressure in the other directions (38, 40). The diaphragm also impinges on the left lateral side and may have some contribution to the proximal part (27), but its striated muscle would not normally be responsive to muscarinic blockade. On the other hand, at rest, circular muscle maintains its intrinsic tone in the LES and appears little affected by cholinergic excitation but modulated by nitricergic inhibition in some species such as the dog (44) and

Fig. 2. LES muscle motor responses to EFS at varied frequencies with Nω-nitro-L-arginine (L-NNA; 10^{-4} M) ± atropine (10^{-6} M). LES sling muscle (A) can relax with NO; there is no significant non-NO relaxation (n = 20). Circular muscle (B) has a small cholinergic contraction and both NO and non-NO relaxations (n = 20). Basal resting tone is lower in the sling muscle.
perhaps the opossum (36, 53) but probably not in the human (12) and cat (52). It is likely that the circular muscle and its myogenic tone is responsible for the resting pressure in the remainder of the sphincter circumference and for the increase in pressure in those species where it occurs with NOS blockade in vivo. In vitro, our present study in the cat and that of González et al. (11) of the human circular LES muscle support these observations.

In vivo, NO is the neurotransmitter primarily responsible for active inhibition of the LES (12, 36, 52, 53). However, the LES tone in vivo that is maintained by cholinergic vagal excitation of the sling component could be readily reduced by switching off this excitation (15, 26). Our studies demonstrated that the sling is normally responsive to NO, and there is also some limited capacity for active vagally driven nitrergic action to facilitate relaxation of the sling. This neural capacity was functionally less evident in the sling. In the sling, there was also some suggestion of a non-NO component to the inhibition in the cat, seen at higher frequencies of stimulation. Studies in the sling muscle in the guinea pig have indicated the presence of a NO and a small non-NO inhibition in this species (54). However, immunohistochemical identification of the neurons in the sling demonstrated that 79% of the neurons were cholinergic and distributed close to or on the sling muscle. Only 15% of the sling neurons were positive for NOS and therefore inhibitory. In contrast, the circular muscle has 53% of the local motor neurons positive for NOS and only 47% of the neurons are cholinergic. Furthermore, 86% of the esophageal neurons sending innervation to the LES circular muscle are also nitrergic (3). The esophageal body sends virtually no neural input to the sling muscle. Therefore, the active neural inhibitory mechanism is much less evident in the sling, both physiologically and neuroanatomically. However, this mechanism could act to facilitate or replace the relaxation effected by switching off its cholinergic excitation. The sling muscle has the ability to fully relax to NO when its tone is elevated by cholinergic excitation, as shown by our experiments with betahanechol stimulation of the muscle.

Previous studies (7, 11, 16, 24, 31, 39, 48, 54) along with our present findings indicate that the circular muscle in vitro is readily relaxed by neural release of NO. In both the cat and human, there is also a slow relaxation due to the presence of a non-NO inhibitor seen at higher frequencies in the cat and with a high nicotine concentration in the human (11). González et al. (11) proposed that this slow relaxation is due to ATP release, although others have provided evidence for vasoactive intestinal polypeptide (VIP) and other substances as potential mediators (1–3, 20, 34, 42, 47–50). For example, it has been reported that NOS- and VIP-immunoreactive nerves were often found to display colocalization in the cat LES (32, 33). We did not explore the nature of the mediator of this relaxation. Its physiological relevance is also unknown, although its presence is most evident at higher frequencies that are not in the usual physiological range of 1–10 Hz (10, 26, 41). Perhaps it could serve as a compensatory role in pathological conditions where...
NO is absent, such as in achalasia (14, 25). In human circular muscle, nicotine has little or no cholinergic excitatory effect compared with that induced by EFS when NO- and non-NO-induced relaxation is blocked (11), supporting the concept that the high resting myogenic tone of the LES circular muscle tone is little augmented by cholinergic excitation under normal circumstances while the inhibitory innervation is dominant.

The tension range covered by the two LES muscles is very similar. In vivo, this allows LES resting tone to be maintained throughout the entire circumference of the sphincter, slightly higher on the left lateral side, due to the vagal cholinergic excitation of the sling. Although the mechanisms by which both LES muscles relax may be completely different, with relaxation of the sling muscle likely occurring predominantly by switching off the cholinergic excitatory influence and the circular muscle relaxing predominantly due to release of NO, both muscles will relax to approximately the same tension.

Knowledge of these regional differences in LES sling and circular muscle has obvious clinical and therapeutic implications. For example, the sling contraction could protect against reflux. Those factors that increase the circular muscle may be all that is necessary to relieve the high tone circular muscle (14, 25) results in LES pressures equalizing around the sphincter circumference to the resting myogenic tone of the LES circular muscle (14, 25) results in LES pressures equalizing around the sphincter circumference to the high tone circular muscle (14, 25). Reduction of LES pressure in vivo with the administration of nifedipine would presumably involve primarily the cholinergic innervation of the sling muscle (35) and suggests that the injection should be directed mainly to the left. The regional differences between sling and circular muscles also provide different options for the surgical management of achalasia (17, 23, 28, 45, 51). Cutting the circular muscle may be all that is necessary to relieve the functional obstruction while leaving the cholinergic sling activity will protect against reflux. Those factors that increase sphincter pressure would have the opposite clinical and therapeutic implications. For example, the sling contraction could be augmented by pharmacological manipulation of its cholinergic control and give credence to the use of cholinergic agonists in raising LES pressure for treatment of gastroesophageal reflux (8). Similarly, attention could be directed primarily to the sling in the surgical management of reflux (18). Significant attention has been directed to pharmacological manipulation of the nitrergic control of the transient lower esophageal sphincter relaxations (TLESR) in patients with reflux, presumably directed primarily to the circular muscle although the role of the sling in the TLESR is unknown (13).


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GRANTS

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