Evaluation of nitric oxide as an inhibitory neurotransmitter in the equine ventral colon

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Objective—To determine the role of nitric oxide and an apamin-sensitive nonadrenergic noncholinergic inhibitory neurotransmitter on contractility of the ventral colon of horses. Sample population—Strips of the circular and longitudinal muscle layers and taenia of the ventral colon from 14 horses. Procedure—Muscle strips were suspended in tissue baths and attached to force transducers. Contractile activity of circular, longitudinal, and taenia muscle strips in response to electrical field stimulation was measured after addition of apamin and a nitric oxide inhibitor, N-nitro-L-arginine methyl ester (L-NAME). Results—Electrical field stimulation reduced contractile activity in the circular muscle layer and taenia but not the longitudinal muscle layer. Addition of L-NAME significantly reduced inhibitory contractile activity at all frequencies for the circular muscle layer, whereas a significant effect was evident for the taenia only at the highest frequency. The combination of L-NAME and apamin resulted in a significant reduction in inhibitory activity of the taenia at all frequencies but for circular muscle only at lower frequencies. Conclusions and Clinical Relevance—Nitric oxide and an apamin-sensitive neurotransmitter appear to mediate a component of inhibitory transmission in the circular muscle and taenia, but not the longitudinal muscle layer, of the equine ventral colon. Nitric oxide has a role in regulating contractile activity of the equine ventral colon, and nitric oxide synthase inhibitors may be useful in horses with ileus of the large colon. (Am J Vet Res 2000;61:64–68)

In horses, motility disturbances of the gastrointestinal tract commonly manifest as colic and represent an important cause of mortality and morbidity. In a retrospective study,1 1% of the large colon accounted for approximately a third of the affected horses in which a cause was identified. Motility disturbances may contribute to generalized adynamic ileus after abdominal surgery or peritonitis, large intestinal impaction, displacement, and torsion. Although most causes of large-colon disease remain obscure, poor-quality feed, advanced age, debilitation, poor dentition, luminal parasites, restricted exercise or change in amount of activity, and abnormal motility patterns are likely contributing factors.1 Gastrointestinal motility is regulated by an intricate interaction between neural and humoral components with input from the central, autonomic, and enteric nervous systems. In the large colon, the enteric nervous system and various hormones regulate luminal and electrolyte transport, with fine-tuning contributed locally by prostaglandins, cytokines, and other intestinal peptides.2 The extrinsic and intrinsic components of the enteric nervous system appear to be primary mechanisms involved in the general regulation of motility, and administration of anticholinergic drugs such as atropine can delay gastric emptying by inhibiting propulsive contractile activity.3

Gastrointestinal motility is also believed to be regulated via intrinsic inhibitory and excitatory neurons known as nonadrenergic-noncholinergic (NANC) neurons.4 The primary inhibition of gastrointestinal tract motility is believed to be mediated by NANC neurons.5 The role of these NANC neurons has been evaluated in humans, cats, sheep, rodents, and horses.6–8 After inhibiting adrenergic and cholinergic nerves, stimulation of NANC neurons located within myenteric plexus ganglia resulted in relaxation of the smooth muscle in these species, likely via a nitric oxide (NO)-dependent mechanism that exerts an inhibitory effect by hyperpolarizing smooth muscle cells.9,10

The predominant NANC inhibitory neurotransmitters that are believed to be involved in regulation of colonic motility include vasoactive intestinal peptide, ATP, and NO.6,11 It is likely that >1 transmitter regulates smooth muscle contractile activity. The action of NANC neurons in the circular layer of some species have been divided into apamin-sensitive, L-N-nitro-arginine-sensitive, and apamin-L-N-nitro-arginine-resistant mechanisms.12 Apamin, a derivative of bee venom, participates in the inhibitory response by blocking small-conductance calcium-activated potassium channels and blocking the activity of a neurotransmitter suspected to be ATP.13

Nitric oxide has been implicated in the regulation of motility in various species.3 However, the contribution NO makes to gastrointestinal tract motility varies by species and segment of intestinal tract examined. In horses, NO and an apamin-sensitive substance are involved in regulating the inhibitory activity of the jejunal circular muscle.8 To evaluate the effect of NO on contractile activity of the ventral colon, N-nitro-L-arginine methyl ester (L-NAME), an arginine analogue and selective NO synthase inhibitor, was evaluated to determine its effects on the circular and longitudinal muscle layers and taenia of the ventral colon. Furthermore, the mechanism of action of apamin in this preparation was investigated in tissue previously treated with ATP.

Materials and Methods

Tissue samples—Specimens were collected from 14 horses euthanatized for reasons unrelated to the gastroin...
to the baths to inhibit muscarinic and adrenoceptors, respectively. Additions of each were added to the baths to inhibit muscarinic and adrenoceptors, respectively. 

Functional studies—Tissue specimens were pinned flat, using a slight amount of tension, in dissecting dishes containing sufficient Krebs buffer solution to ensure the tissues were completely immersed. After removal of the mucosa, strips measuring 2 × 10 mm were cut in a direction oriented parallel to the various muscle layers (circular, longitudinal, taenia). Each of the muscle strips was mounted on glass hooks in a tissue bath system (20-ml capacity), which contained oxygenated Krebs buffer solution warmed to 37.5 ± 0.2°C, as described elsewhere. The other end of each muscle strip was connected to a polygraph chart recorder that measured tension by means of force transducers. After equilibrating in the baths for 45 minutes, muscle strips were stretched to achieve a tension force of 2 g and allowed to equilibrate for an additional 60 minutes. At that time, atropine and guanethidine (1 M solutions of each) were added to the baths to inhibit muscarinic receptors and adrenoceptors, respectively. Substance P (2 × 10–5 M) was added to generate consistent contractile activity for evaluation of the effect of inhibitory neurons. On the basis of preliminary studies, this concentration of substance P was selected as the minimal amount required to elicit a consistent contractile pattern. At 20-minute intervals, the baths were flushed with fresh Krebs buffer solution containing atropine and guanethidine. After the equilibration period, electrical field stimulation was administered by use of platinum ring electrodes attached to a stimulator, using frequencies of 1, 2, 5, 10, and 20 Hz (1-minute series of 0.5-ms pulses at 60 V). In preliminary studies, stimulation at 20 Hz produced maximal contractile inhibition. For each stimulation frequency, magnitude of contractile inhibition was obtained by comparing the contractile activity during electrical stimulation after the addition of L-NAME, apamin, and the combination of L-NAME and apamin to the contractile activity during electrical stimulation without addition of these agents.

Effects of L-NAME and apamin were evaluated on the inhibitory response generated by electrical field stimulation. Specifically, tissue strips were initially stimulated at the previously mentioned frequencies and flushed with Krebs buffer solution. Then, L-NAME (200 μM) was added to the baths and allowed to equilibrate for 15 minutes. Tissue strips were then stimulated again at frequencies of 1 to 20 Hz. After flushing with Krebs buffer solution, L-NAME (200 μM) and apamin (0.3 μM) were added to the tissue baths, and after a 15 minute equilibration, tissue strips were stimulated again. To determine the effect of apamin, the same protocol was followed, using apamin first, followed by L-NAME. Effect of the agents was expressed as percentage change from control activity.

To evaluate the mechanism of action of apamin on the circular and longitudinal muscle layers and taenia, ATP (4 × 10–5 M) was added to the muscle strips, and contractile activity was recorded after 10 minutes of incubation. After addition of ATP, apamin (0.3 μM) was added, and contractile activity was recorded at 10 minutes. For strips of taenia, apamin was added to muscle strips without the addition of ATP to determine whether the effect of apamin on contractility was repeatable without a purinoceptor agonist. All agents were purchased in crystalline form from a commercial vendor, and fresh solutions were prepared on the day of the study. All solutions added to the tissue baths were prepared in Krebs buffer solution.

Data analyses—Paired Student t-tests were used to compare the percentage of inhibition during electrical field stimulation with and without addition of L-NAME and apamin. Degree of inhibition detected during electrical field stimulation was calculated by measuring the area under the contractile curve 1 minute before stimulation, compared with the area under the contractile curve during the period of stimulation (2-minute interval) at each frequency, as described elsewhere. To evaluate the effect of apamin on ATP-treated tissue, a paired Student t-test was used to compare contractile activity after addition of ATP and after addition of the combination of ATP-apamin with baseline contractions. A value of P < 0.05 was considered significant.

Results

Baseline contractility—Contractile activity of muscle strips from the taenia and circular smooth muscle layers was inhibited during the stimulation period at all stimulation frequencies tested, as evidenced by a decrease in the amplitude and frequency of contractions (Figs 1 and 2). At the lowest frequency (1 Hz), a slight contractile activity was evident during the latter portion of stimulation. However, during stimulation at higher frequencies (5, 10, and 20 Hz), all contractile activity was completely inhibited in the circular muscle strips. In most taenia strips, some contractile activity was evident, although the
frequency and amplitude of contractions were profoundly reduced. At 20 Hz, the taenia strips had an increase in frequency and amplitude of contractions. Contractile activity of the longitudinal muscle strips was unaffected by electrical field stimulation. For circular and taenia muscle strips, cessation of electrical field stimulation was followed by a rebound or increase in amplitude, which was followed by a return to phasic contractions that were similar to the prestimulation contractile activity.

Effect of L-NAME and apamin—For circular muscle strips, addition of L-NAME (200 µM) significantly \((P < 0.001)\) reduced inhibition of contractile activity at all stimulation frequencies (Fig 3). Amplitude and frequency of contractions of taenia were significantly \((P = 0.004)\) reduced at a stimulation frequency of 20 Hz. At a stimulation frequency of 10 Hz, the percentage of inhibition during stimulation after addition of L-NAME tended to be less, but not significantly so, than at frequencies of 1, 2, and 5 Hz. For the longitudinal muscle layer, addition of L-NAME did not affect contractile activity during electrical field stimulation. For circular muscle strips after addition of apamin alone, a significant reduction of inhibition was evident during electrical field stimulation only at frequencies of 1 \((P = 0.01)\) and 20 \((P = 0.04)\) Hz. For taenia, a significant \((P = 0.04)\) reduction of inhibitory activity was detected at all frequencies. The combination of L-NAME and apamin resulted in a significant reduction in inhibition during stimulation at all frequencies for taenia and at 1, 2, and 5 Hz for circular muscle strips; there was not a significant effect on contractile activity for longitudinal muscle strips. The combination of L-NAME and apamin appeared to have an additive effect, as indicated by an increase in frequency and amplitude of contractions, although greater inhibition of contractile activity was detected at lower frequencies (1 and 2 Hz), compared with higher frequencies (5, 10, and 20 Hz), for circular smooth muscle and taenia strips (Fig 1).

After the addition of ATP and apamin to the longitudinal muscle strips \((n = 8)\), we did not detect a significant effect on frequency or amplitude of contractile activity. For the circular muscle layer \((n = 12)\), addition of ATP resulted in a significant \((P < 0.001)\) reduction in contractile activity, compared with baseline contractility, whereas the addition of apamin resulted in a significant \((P < 0.001)\) increase in contractile activity. For the taenia \((n = 23)\), a significant effect was not detected after the addition of ATP, although there was a pattern of reduced contractile activity. The addition of apamin resulted in a significant \((P < 0.001)\) increase in contractile activity, with or without the addition of ATP.

Discussion
Nitric oxide affects intestinal contractility predominantly via an inhibitory effect.\(^{12,13}\) Analysis of results of the study reported here suggested that NO regulates inhibitory contractile activity of the equine ventral colon, specifically the taenia and circular smooth muscle.
layers. This is consistent with results of a study in which the activity of NO on equine jejunum was investigated. In the circular layer of the small intestine, exogenous NO gas and a NO donor (sodium nitroprusside) inhibited contraction, whereas a NO synthase inhibitor (L-NAME) inhibited the reduction in contractility during electrical field stimulation. The circular layer of the ventral colon responded in a manner similar to the effect observed in the small intestine. In contrast, the longitudinal layer was not affected by electrical field stimulation, NO inhibition, or ATP and apamin, whereas the taenia was affected intermediately. The finding that low frequencies of stimulation did not completely result in total inhibition of contractile activity is likely attributable to frequency-dependent release of NO.

Differences in activity between circular and longitudinal muscle strips have been observed in other contractility studies. It is known that circular muscle of horses is more sensitive to tachykinins, relative to the longitudinal muscle layer, regardless of the intestinal segment evaluated. This difference likely is a result of variations in receptor specificity and density in the circular and longitudinal muscle layers. Although immunochemical studies in guinea pigs and many other species documented that there is NO synthase activity in the circular and longitudinal muscles, it is possible that lack of response to electrical field stimulation in the equine longitudinal muscle layer is attributable to a lack of NO synthase. A predominance of NO synthase activity in the circular muscle layer, relative to the longitudinal layer, has also been described for the colon in humans. Although NO is involved in inhibitory neurovascular control of the longitudinal muscle of the colon in dogs and humans, the longitudinal layer of horses apparently lacks substantial NO activity.

In the study reported here, confounding influences of cholinergic and adrenergic neurotransmitters were minimized by adding atropine and guanethidine to the muscle baths. Electrical field stimulation was selected as the method to evaluate NO activity, because other studies confirmed that nitric oxide is liberated from all portions of the gastrointestinal tract after electrical field stimulation. Support that NO mediates a component of the inhibitory response to electrical field stimulation in the circular and taenia muscles is evident by the finding that contractile activity was restored after addition of the NO synthase inhibitor L-NAME. In the small intestine of horses, addition of L-NAME during electrical field stimulation partially restores contractile activity; however, inhibitory activity was only completely reversed after addition of apamin. Therefore, apamin likely induces smooth muscle contraction by blocking calcium-dependent potassium channels in the smooth muscle and potentially inhibiting ATP-induced hyperpolarization. In the longitudinal muscle layer of horses, ATP and the apamin-sensitive neurotransmitters do not appear to have a major role in regulating intestinal motility. In contrast, the contractile activity of the circular muscle layer of the horses in our study was inhibited by ATP and restored after addition of apamin, suggesting that ATP is a mediator of the apamin-sensitive mechanism, and that apamin is likely acting via inhibition of purinergic receptors. In the taenia, although the addition of ATP tended to reduce contraction, this was not a significant effect. However, the addition of apamin did result in a significant increase in contractile activity, relative to baseline activity. It is possible that, in the taenia, apamin may operate via another mechanism than blockade of purinergic receptors, especially because the effect on contractility was the same with and without the addition of ATP. It is believed that ATP inhibits contractile activity via hyperpolarization of the membrane through an increase in intracellular calcium and activation of calcium-dependent potassium channels, thereby acting as a purinoceptor agonist. Further investigations to evaluate the mechanism of action of apamin in this location of the gastrointestinal tract are warranted.

In the preparation used in this study, the increase in contractility after addition of L-NAME and apamin in the circular and taenial muscle strips indicated that there was an underlying inhibitory tone that was apamin-sensitive and had a NO component. In the colon, effects of L-NAME and apamin were additive, which suggested that inhibition of smooth muscle activity may involve an apamin-sensitive as well as a NO component.

Although analysis of our results suggests that NO is the predominant neurotransmitter involved in inhibiting contractile activity of the circular layer of the colon, additional factors may be active, because inhibition of NO synthase did not completely restore contractile activity. The concentration of L-NAME used makes the possibility of incomplete enzymatic inhibition unlikely. In other species, NO synthase is coexpressed with various neuropeptides (eg, vasoactive intestinal peptide), although NO is believed to play the predominant role.

In the equine ventral colon, the taenia are discrete bands of smooth muscle and connective tissue that comprise the longitudinal portion of the tunica muscularis and contribute to intestinal contractility. It is possible the taenia may possess less NO synthase activity, compared with NO synthase activity in the circular layer, because the addition of L-NAME was only effective at the highest frequency. Potentially, the taenia may contain additional neurotransmitters (specifically, an apamin-sensitive neurotransmitter) that would account for the significant increase in contractile activity after addition of apamin alone or the combination of apamin and L-NAME.

Intestinal ileus associated with inflammation and subsequent release of endogenous NO from inflammatory cells can lead to motility disorders. Systemic administration of NO synthase inhibitors may result in attenuation of ileus. Experimentally induced chronic ileitis was reduced after administration of NO synthase inhibitors. The finding that NO inhibits contractile activity in the circular layer and partially in the taenial band suggests that use of NO synthase inhibitors may have a role in the treatment of horses with motility dysfunction.

Model 70 polygraph, Grass Astromed, West Warwick, RI.
1FT03, Grass Astromed, West Warwick, RI.
2Sigma Chemical Co, St Louis, Mo.
3RI588, Grass Astromed, West Warwick, RI.

References


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