Adrenergic, cholinergic, and nonadrenergic-noncholinergic intrinsic innervation of the jejunum in horses

Erin D. Malone, DVM, PhD; Mathur S. Kannan, BVSc, PhD; David R. Brown, PhD; Tracy A. Turner, DVM, MS; Ava M. Trent, DVM, MS

Objective—To determine the major neurotransmitters that regulate contractile activity in the jejunum of horses.

Sample Population—Jejunal specimens from 65 horses without gastrointestinal tract lesions.

Procedure—Jejunal smooth muscle strips, oriented in the plane of the circular or longitudinal muscular layer, were suspended isometrically in muscle baths. Neurotransmitter release was induced by electrical field stimulation (EFS) delivered at 30 and 70 V intensities and at various frequencies on muscle strips maintained at low or high muscle tone. To detect residual nonadrenergic-noncholinergic neurotransmission, the response of muscle to EFS in the presence of adrenergic and cholinergic blockade was compared with the response in the presence of tetrodotoxin.

Results—Atropine (ATR) decreased the contractile response of muscle strips to EFS under most conditions. However, ATR increased the contractile response of high-tone circular muscle. Adrenergic blockade generally increased the muscle responses to 30 V EFS and in high-tone longitudinal muscle but decreased contractile responses in high-tone circular muscle. Tetrodotoxin significantly altered the responses to EFS, compared with adrenergic and cholinergic receptor blockade.

Conclusions—Acetylcholine and norepinephrine appear to be important neurotransmitters regulating smooth muscle contractility in the equine jejunum. They induce contraction and relaxation, respectively, in most muscle preparations, although they may cause opposite effects under certain conditions. In addition, nonadrenergic-noncholinergic excitatory and inhibitory influences were detected.

Clinical Relevance—Acetylcholine or norepinephrine release within the myenteric plexus of horses may alter gastrointestinal motility. (*Am J Vet Res* 1999;60: 898–904)

Most motility modifiers available for use in horses are designed to alter the level of nonadrenergic or cholinergic tone within the intestinal tract. Many of these have been found to be only minimally effective, or their use is fraught with serious adverse effects.[1-3]

Received Aug 17, 1998.
Accepted Nov 16, 1998.

From the Departments of Clinical and Population Sciences (Malone, Turner, Trent) and Veterinary Pathobiology (Kannan, Brown), College of Veterinary Medicine, University of Minnesota, St Paul, MN 55108.

This report represents a portion of a thesis submitted by the senior author to the graduate school as partial fulfillment of the requirement for the PhD degree.

Recent research in other species has indicated that **nonadrenergic-noncholinergic** (NANC) neurons of the enteric nervous system also can serve as mediators of **gastrointestinal** (GI) excitatory and inhibitory responses, through regulation of the peristaltic reflex.[4] The pharmacologic control of NANC neurons eventually may allow more effective modification of gastrointestinal motility in horses. However, to the authors' knowledge, the amount of NANC activity in the GI tract of horses has not been defined. Definition of NANC activity, in turn, requires in-depth knowledge of intrinsic adrenergic and cholinergic activity.

Several in vivo and in vitro studies have been performed to document the responsiveness of the GI tract of horses to adrenergic and cholinergic mediators. Parasympathomimetic agents have been found to directly stimulate motility in horses, and parasympatholytic agents are capable of inducing inhibition of motility and delay in transport.[1,5-12] In general, selective α_1- and β-adrenergic receptor agonists inhibit intestinal activity in horses, as do drugs with documented activity at adrenergic receptors.[1,6,8,11,13] **Norepinephrine** (NE) and α_2-adrenergic agonists are also capable of temporarily stimulating longitudinal muscle activity.[12,14,15] However, despite the extensive research in this field, studies have not documented the release of **acetylcholine** (ACH) or NE from neurons within the myenteric plexus or determined the response of the GI tract smooth muscle to their release at that level. Additionally, conflicting results have been published on inhibitory sympathetic tone in the GI tract of horses.[1,12] Finally, there is evidence that alterations in the function of ACH or NE, or both, may develop in equids with grass sickness, endotoxemia, and postoperative ileus.[1,16-19]

The study reported here was designed to determine the magnitude of adrenergic, cholinergic, and NANC modulation of jejunal smooth muscle contractility in vitro and provide a reference point with which samples collected from horses with colic or intestinal dysfunction can be compared to assess potential alterations in neural regulation of intestinal motility. On the basis of previous research in horses, we hypothesized that blockade of cholinergic neurotransmission would decrease the response to depolarization of myenteric neurons by **electrical field stimulation** (EFS) significantly, but that blockade of adrenergic neurotransmission would have minimal effect, and that evidence of NANC neurotransmission could be found within normal jejunal smooth muscle of horses.

serum from healthy cats. Two different diets were chosen, because composition of the foodstuff may affect gastric and intestinal phases of exocrine pancreatic secretion.[7] Diet 1 had high protein and fat contents, whereas diet 2 had protein and fat contents comparable to those of many feline maintenance diets. Cats were offered food for only 1 hour to simulate conditions under which a clinical patient would consume a meal.

The lack of a significant difference in serum fTLI between periods was expected, given that cats were fed each diet for 10 days before blood samples were collected. The significant difference in fTLI in serum among the 6 cats was not surprising. Likewise, the significant difference between groups was not unexpected and was most likely a result of the small sample size (3 cats/group). A significant difference in serum fTLI was not found over time; however, because mean serum fTLI was increased slightly for both diets 1 hour after cats were fed, compared with the baseline values, we elected to compare values for these times by use of one-sided paired *t*-tests. This revealed a statistically significant increase (mean increase, 1.7 µg/L) in serum fTLI 1 hour after cats were fed diet 2. However, because the reference range for fTLI in serum is 17 to 49 µg/L, this increase was judged to be clinically unimportant. In contrast, the increase 1 hour after cats were fed diet 1 (mean increase, 3.1 µg/L) was not statistically significant, probably because of the small sample size. However, even if it had been statistically significant, it also would have been judged to be clinically unimportant.

The study reported here did not answer the question of whether feeding would affect fTLI in serum from cats with disorders of the exocrine portion of the pancreas. More specifically, it did not answer the question of whether cats with exocrine pancreatic insufficiency that were fed before blood samples were collected may have serum fTLI in the questionable range (8 to 17 µg/L) or even within the reference range (17 to 49 µg/L) and, therefore, escape diagnosis. Until such data have been collected, it seems prudent to maintain the recommendation that food be withheld from cats before blood samples are collected for determination of fTLI in serum. However, given that in the study reported here, values were increased only 1 hour after cats were fed, it would seem that withholding food for 3 to 4 hours before collecting blood samples should be sufficient.

[a]Washabau RJ, Callan MB, Williams DA, et al. Cholecystokinin secretion is preserved in canine pancreatic insufficiency (abstr). *J Vet Int Med* 1995;9:193.
[b]CNM feline CV canned, Ralston Purina Co, St Louis, Mo.
[c]CNM feline UR dry, Ralston Purina Co, St Louis, Mo.
[d]N6, Diagnostic Products Corp, Los Angeles, Calif.
[e]SAS System, SAS Institute Inc, Cary, NC.
[f]Spillmann T. Zur Diagnostik der exokrinen Pankreasinsuffizienz beim Hund. Dr med vet Thesis, Medizinische und Gerichtliche Veteriärklinik I der Justus-Liebig-Universität, Giessen, Germany, 1995.

References

1. Steiner JM, Medinger TL, Williams DA. Development and validation of a radioimmunoassay for feline trypsin-like immunoreactivity. *Am J Vet Res* 1996;57:1417–1420.
2. Steiner JM, Williams DA. Feline trypsin-like immunoreactivity in feline exocrine pancreatic disease. *Compend Contin Educ Pract Vet* 1996;18:543–547.
3. Steiner JM, Williams DA. Feline exocrine pancreatic disorders: insufficiency, neoplasia, and uncommon conditions. *Compend Contin Educ Pract Vet* 1997;19:836–849.
4. Williams DA, Batt RM. Sensitivity and specificity of radioimmunoassay of serum trypsin-like immunoreactivity for the diagnosis of canine exocrine pancreatic insufficiency. *J Am Vet Med Assoc* 1988;192:195–201.
5. Steiner JM, Williams DA. Feline pancreatitis. *Compend Contin Educ Pract Vet* 1997;19:590–603.
6. Williams DA, Batt RM. Exocrine pancreatic insufficiency diagnosed by radioimmunoassay of serum trypsin-like immunoreactivity in a dog with a normal BT-PABA test result. *J Am Anim Hosp Assoc* 1986;22:671–674.
7. DiMagno EP, Layer P. Human exocrine pancreatic enzyme secretion. In: Go VLW, DiMagno EP, Gardner JD, et al, eds. *The pancreas: biology, pathobiology, and disease*. 2nd ed. New York: Raven Press, 1993;275–350.
8. Chey WY. Hormonal control of pancreatic exocrine secretion. In: Go VLW, DiMagno EP, Gardner JD, et al, eds. *The pancreas: biology, pathobiology, and disease*. 2nd ed. New York: Raven Press, 1993;403–424.
9. Singer MV. Neurohormonal control of pancreatic enzyme secretion in animals. In: Go VLW, DiMagno EP, Gardner JD, et al, eds. *The pancreas: biology, pathobiology and disease*. 2nd ed. New York: Raven Press, 1993;425–448.
10. Borgström A, Ohlsson K. Immunoreactive trypsin in serum and peritoneal fluid in acute pancreatitis. *Hoppe-Seyler's Z Physiol Chem* 1978;359:677–681.
11. Borgström A, Ohlsson K. Immunoreactive trypsins in sera from dogs before and after induction of experimental pancreatitis. *Hoppe-Seyler's Z Physiol Chem* 1980;361:625–631.
12. Borgström A. The fate of intravenously injected trypsinogens in dogs. *Scand J Gastroenterol* 1981;16:281–287.
13. Borgström A, Ohlsson K. Studies on the turnover of endogenous cathodal trypsinogen in man. *Eur J Clin Invest* 1978;8:379–382.

Materials and Methods

Preparation of tissues—Intestinal specimens were obtained from horses without GI tract disorders or systemic disease that were anesthetized or recently (within 30 minutes) euthanatized by IV administration of an overdose of pentobarbital. A length of the middle portion of the jejunum was excised, cut along the mesenteric border, and placed in ice-cold oxygenated Krebs-bicarbonate buffer (composition [mM]: Na^+, 143; K^+, 5.9; Cl^-, 134.1; Ca^{2+}, 2.5; Mg^{2+}, 1.2; HCO_3^-, 21.0; $H_2PO_4^{2-}$, 1.2; and dextrose, 10.0; pH 7.4). The mucosa and submucosa were removed by sharp dissection. The remaining section of the intestine was cut into 2 × 10-mm strips, oriented in the plane of either the longitudinal or circular smooth muscle. Tissues were allowed to equilibrate for 60 minutes or until a consistent response to field stimulation was obtained.

Electrical field stimulation—The strips of smooth muscle were suspended isometrically under 2 to 4 g of tension (optimal strip tension) between bipolar platinum ring electrodes that were connected to a stimulator.[a] On the basis of results of preliminary studies, the strips were subjected to EFS at intensities of 30 and 70 V and frequencies of 2, 4, 8, and 15 Hz (pulse duration, 1 millisecond; pulse train, 10 seconds). These parameters were chosen to depolarize nerve rather than muscle in the intestine.[b] A resting period of 60 seconds was allowed between each EFS episode for studies done at low (baseline) tone. The EFS also was applied to strips contracted (high tone) with carbachol (10 μM) for most studies and with either histamine (10 to 100 μM) or barium chloride (100 μM) for studies done in the presence of **atropine (ATR)**. Because of the waning of the contraction amplitude, only 15 seconds was allowed between each EFS episode for contracted tissues. A maximum of 2 studies at each level of tone were performed for each muscle strip to avoid fatigue. The order of addition of drugs was randomized throughout the study to minimize changes attributable to fatigue of the muscles. In preliminary studies, 10 μM indomethacin was added to the baths to determine whether there was any evidence of intrinsic or induced prostaglandin release. Alterations were not apparent, and the drug was not included in these studies.

Identification of neurotransmitter release—Prior to EFS, either ATR (1μM) alone, or **propranolol (PRO**; 1 μM) combined with **phentolamine (PTL**; 1 μM) or **yohimbine (YOH**; 0.1 μM), was added to the baths to inhibit cholinergic or nonadrenergic neurotransmission, respectively. Concentrations of indomethacin and these antagonists were chosen to block high amounts of specific agonists on the basis of results in other species and studies done in horses.[11] To identify NANC neuronal activity, adrenergic and cholinergic receptor blockers were added prior to EFS. The neuronal sodium channel blocker **tetrodotoxin (TTX**; 1 μM) was used in a similar manner to verify that neuronal activity remained after blockade of adrenergic and cholinergic receptors as well as to establish neuronal depolarization in response to EFS.

Statistical analyses—Mean response to drugs in a minimum of 6 strips was analyzed for each drug or drug combination. Each comparison was performed only once in tissues from each horse. The EFS studies done at low tone were evaluated in terms of amplitude of contraction at the end of EFS ("on" response) and at 6 seconds after the completion of EFS ("off" response). Other studies in our laboratory did not indicate significant differences in results when the "off" response was analyzed at 3 time points. Because of lack of normal distribution in some data sets, paired data were analyzed by use of the Wilcoxon signed rank test for nonparametric data. Data obtained from study of high-tone strips were evaluated as a fraction of the initial amplitude at 3 time points: 6 seconds after the start of EFS (level A), at the end of EFS (level B), and 6 seconds after completion of EFS (level C; "off" response). These time points were chosen to reflect peak effects for strips at low tone and plateau responses for high-tone muscle strips. One-way ANOVA was used to compare sets of data. For each set of drug studies, comparisons also were made between various stimulus intensities, pulse durations, and repetitions of EFS, using one-way ANOVA for low- and high-tone muscle studies. Statistical significance was set at $P < 0.05$ for all tests. Studies yielding no significant drug effects were evaluated to determine the power of the test (inability to detect a true significant difference because of low numbers of samples).

Results

Longitudinal muscle responses—In strips at low tone (2 to 4 g), 30 and 70 V EFS resulted in a frequency-dependent contractile response for the duration of the stimulation. This contraction peaked at or near the end of the stimulation (**Fig 1**). Atropine inhibited this contractile response at both EFS intensities (**Table 1**). Adrenergic receptor blockade increased the "on" response to 30 V EFS, particularly at low levels of stimulation (2 and 4 Hz), and decreased the "on" response to 70 V EFS (**Table 2**). The effect of adrenergic blockade was visible only in the presence of ATR. For all strips and conditions, responses were similar in either the presence of PTL (α_1- and α_2-adrenoceptor blocker) or YOH (α_2-adrenoceptor blocker).

Longitudinal muscle strips at high tone (mean ± SEM, 9.0 ± 0.3 g) most commonly had a biphasic "on" response to EFS (**Fig 2**). The type of response was similar for all 3 drugs used to contract tissues. Electrical field stimulation initially induced a decrease in the level of tone (level A) that persisted during the first half of stimulation. This was followed by a second phase (level B), which consisted of a gradual increase in tone, generally returning the strips to the original level of tension. Atropine further decreased the amount of muscle contraction during the "on" response in these high-tone muscle strips, but only at 70 V EFS (Table 1).

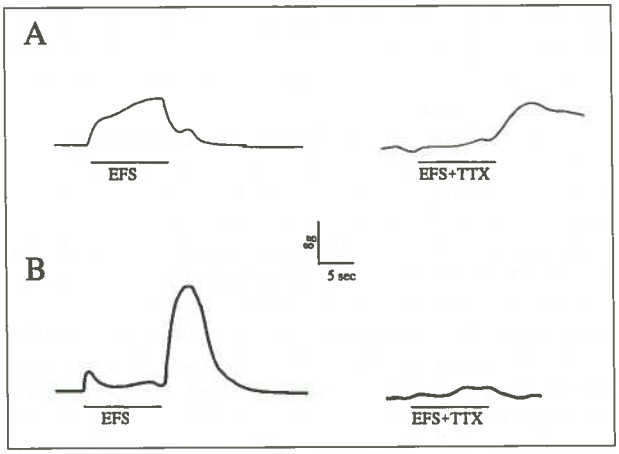

Figure 1—Response to electrical field stimulation (EFS; 70 V, 8 Hz, 1-millisecond pulse duration) of jejunal specimens from 36 horses; specimens were oriented in the plane of the longitudinal (A; n = 34) or circular (B; 36) muscular layer. Horizontal scale marker indicates duration of EFS; vertical scale marker indicates strength of muscle contraction. Notice a response during and after EFS. Addition of 1 μM tetrodotoxin (TTX) significantly ($P < 0.05$) altered both responses. Mean values for peak tensions are depicted in Figure 3.

Table 1—Response (g; mean ± SEM) to atropine (ATR) by longitudinally oriented jejunal strips from horses, after electrical field stimulation at 30 and 70 V

	30 V				70 V			
Variable	n	Control	ATR	*P* value	n	Control	ATR	*P* value
Baseline tension on response*	31	5.66 ± 0.87	2.82 ± 0.74	< 0.01	46	6.94 ± 0.78	5.54 ± 0.78	< 0.01
PC-A	17	1.00 ± 0.03	0.97 ± 0.05	0.55	28	0.91 ± 0.03	0.78 ± 0.04	0.01
PC-B	17	1.01 ± 0.03	0.98 ± 0.06	0.72	28	1.06 ± 0.03	0.90 ± 0.04	< 0.01
Baseline tension off response*	32	3.85 ± 0.83	2.33 ± 0.81	< 0.01	46	5.95 ± 0.77	4.38 ± 0.78	< 0.01
PC-C (off response)	17	1.09 ± 0.04	0.96 ± 0.06	0.09	28	1.23 ± 0.03	1.15 ± 0.05	0.15

*Values for strips at baseline tension given for 8 Hz.
PC-A = Precontracted tissue, level A. PC-B = Precontracted tissue, level B. PC-C = Precontracted tissue, level C. PC strip data given as fraction of mean initial tension for all Hz.

Table 2—Response (g; mean ± SEM) to adrenergic receptor blockade in the presence of ATR by longitudinally oriented jejunal strips, after electrical field stimulation at 30 and 70 V

	30 V				70 V			
Variable	n	Control (ATR)	ATR/PRO/PTL	*P* value	n	Control (ATR)	ATR/PRO/PTL	*P* value
Baseline tension on response*	15	3.72 ± 1.05	5.37 ± 0.94	0.19	16	6.42 ± 1.37	5.18 ± 1.12	0.05
PC-A	9	0.97 ± 0.05	1.22 ± 0.10	0.03	15	0.78 ± 0.04	0.91 ± 0.06	0.06
PC-B	8	0.98 ± 0.06	1.18 ± 0.10	0.09	15	0.90 ± 0.04	1.04 ± 0.06	0.06
Baseline tension off response*	12	1.43 ± 0.82	3.18 ± 1.05	< 0.01	16	5.51 ± 1.55	4.90 ± 1.41	0.40
PC-C (off response)	8	0.96 ± 0.06	0.90 ± 0.11	0.63	15	1.15 ± 0.05	1.19 ± 0.07	0.58

ATR = Atropine. PRO = Propranolol. PTL = Phentolamine.
See Table 1 for key.

Adrenergic blockade in the presence of ATR increased the amount of muscle contraction at level A and less strongly at level B (30 V or lower Hz only; Table 2).

In low- and high-tone longitudinally oriented tissues, the "on" response usually was followed by a separate contractile response that began immediately after cessation of EFS (ie, the "off" response; Figs 1 and 2). Atropine inhibited the contractile "off" response in all studies (Table 1). Adrenergic receptor blockade increased "off" responses of low-tone tissues to 30 V EFS, particularly at low levels of stimulation (2 and 4 Hz; Table 2). This effect of adrenergic receptor blockade was visible only in the presence of ATR. Adrenergic blockade did not affect the "off" response of low-tone tissues to 70 V EFS or of high-tone tissues to either 30 or 70 V EFS (Fig 3).

The sodium channel blocker, tetrodotoxin (TTX), was added to baths to confirm neuronal depolarization and to identify NANC neurotransmission. Tetrodotoxin significantly decreased the contractile response to EFS for all study parameters with the exception of the 30 V "on" response (Fig 1–4). In longitudinally oriented tissues at low tone, ATR and TTX attenuated the contractile "on" response to a similar extent; however, for the "off" response, TTX attenuation was significantly greater than that of ATR (Fig 4). In high-tone tissues, TTX induced a significant decrease in the contractile "off" response, compared with the response to EFS in the presence of adrenergic receptor blockade (mean ± SEM, 1.06 ± 0.08 for TTX, compared with 1.32 ± 0.08 for PRO/PTL). Tetrodotoxin did not increase contractile responses to EFS to any significant degree beyond the effects of adrenergic and cholinergic blockade (Fig 3).

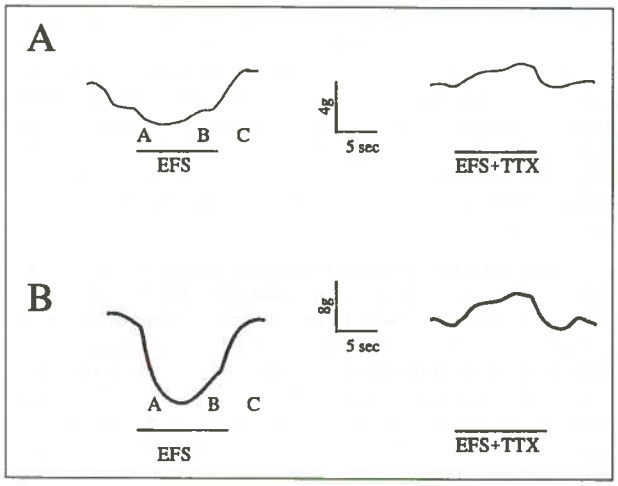

Figure 2—Response to EFS (30 V, 8 Hz, 1-millisecond pulse duration) in high-tone longitudinally (A; n = 34) and circularly (B; 28) oriented jejunal specimens from 32 horses. Horizontal scale marker indicates duration of EFS; vertical scale marker indicates strength of muscle contraction. Notice biphasic response during EFS and a contractile response after EFS. Addition of 1 μ*M* TTX significantly ($P < 0.05$) altered both responses. Mean values for "on" and "off" responses are depicted in Figure 4.

Circular muscle responses—In circularly-oriented muscle strips at low tone, 30 V EFS inhibited spontaneous myogenic activity but caused minimal alterations in basal tone during the stimulation period; however, at 70 V intensity, a contractile response peaking at the end of EFS was apparent (Fig 1). Atropine inhibited the contractile "on" response to 70 V EFS but did not alter the pattern of minimal activity observed during 30 V EFS (Table 3). Adrenergic receptor blockade did not affect the "on" response to EFS at either intensity (Table 4).

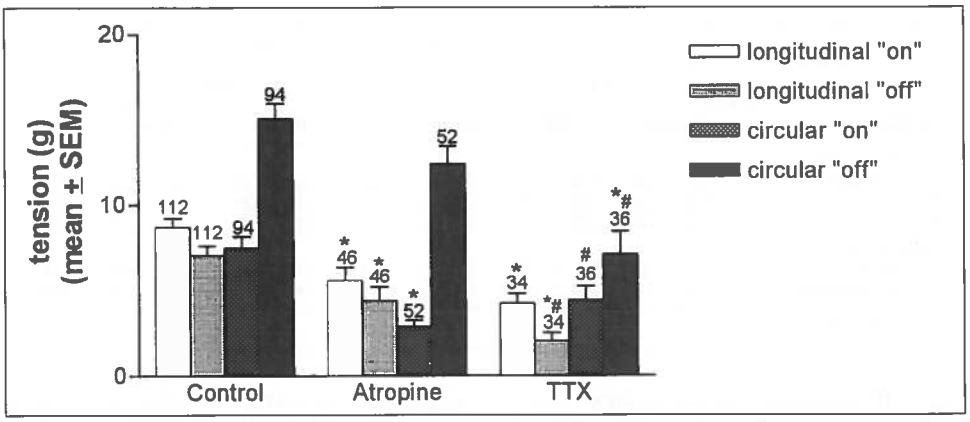

Figure 3—Noncholinergic excitatory activity in low-tone circularly and longitudinally oriented jejunal specimens from horses. Atropine caused a significant decrease in contractile activity, but TTX was able to further inhibit the contractile response to EFS (70 V, 8 Hz). ★ = Significant ($P < 0.05$) difference from response for control specimens. # = Significant difference from response for specimens incubated with atropine. Numbers above bars indicate sample size.

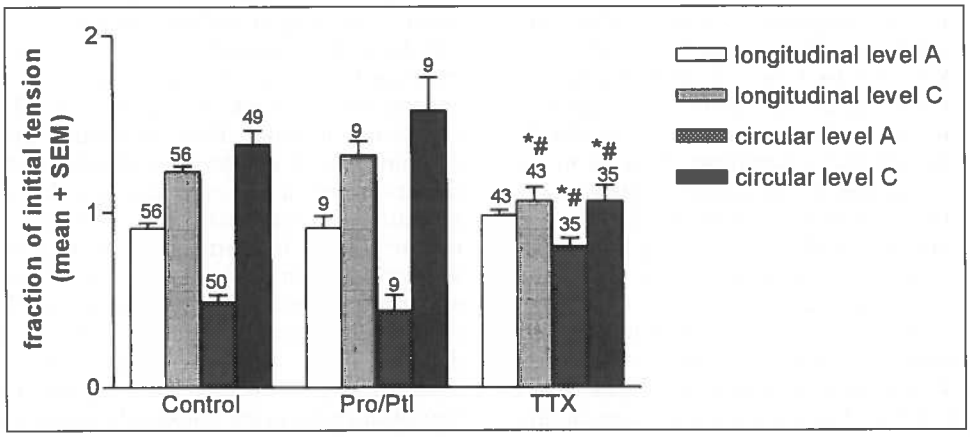

Figure 4—Nonadrenergic inhibitory activity in high-tone circularly and longitudinally oriented jejunal specimens from horses. Propranolol and phentolamine (Pro/Ptl) minimally affected response to EFS (70 V; mean values for all Hz) in the presence of atropine. Tetrodotoxin significantly ($P < 0.05$) increased the contractile "on" response to EFS in circularly oriented specimens, compared with combined adrenergic and cholinergic receptor blockade. ★ = Significant difference from response for control specimens. # = Significant difference from response for specimens incubated with Pro/Ptl. Numbers above bars indicate sample size.

Table 3—Response (g; mean ± SEM) to ATR by circularly oriented jejunal strips, after electrical field stimulation at 30 and 70 V

	30 V				70 V			
Variable	n	Control	ATR	*P* value	n	Control	ATR	*P* value
Baseline tension on response*	39	0.37 ± 0.12	0.80 ± 0.36	0.46	52	6.12 ± 0.79	2.88 ± 0.34	< 0.01
PC-A	24	0.50 ± 0.05	0.43 ± 0.06	0.39	34	0.49 ± 0.04	0.42 ± 0.05	0.28
PC-B	24	0.57 ± 0.08	0.60 ± 0.08	0.77	40	0.65 ± 0.06	0.86 ± 0.06	0.01
Baseline tension off response*	39	12.28 ± 1.07	10.30 ± 1.07	0.21	52	15.36 ± 1.06	12.38 ± 1.06	0.23
PC-C (off response)	24	1.30 ± 0.10	1.74 ± 0.11	< 0.01	34	1.38 ± 0.08	1.46 ± 0.10	0.56

See Table 1 for key.

In high-tone (mean ± SEM, 13.8 ± 0.3 g) circularly oriented muscle strips, the response was similar to that of the longitudinally oriented muscle; however, the contractile and relaxant responses generally were more exaggerated (deviated further from original level of tension; Fig 2 and 3). Additionally, circular muscle did not respond as well to histamine as a contraction agent. In these high-tone strips, ATR increased the contractile response to 70 V EFS (Table 3). Adrenergic blockade did not affect specimens subjected to 30 V EFS; however, for 70 V EFS, adrenergic blockade in the presence of ATR decreased the contractile "on" response (Table 4)

Similar to longitudinally oriented muscle strips exposed to EFS, a strong contractile response immediately followed the cessation of field stimulation in circularly oriented strips (Fig 1 and 2). Atropine inhibited the contractile "off" response in tissues at low tone.

Table 4—Response (g; mean ± SEM) to adrenergic receptor blockade in the presence of ATR by circularly oriented jejunal strips, after electrical field stimulation at 30 and 70V

	30 V				70 V			
Variable	n	Control	ATR	*P* value	n	Control	ATR	*P* value
Baseline tension on response*	16	1.21 ± 0.78	0.81 ± 0.38	0.67	23	3.02 ± 0.46	3.23 ± 0.52	0.91
PC-A	9	0.43 ± 0.06	0.39 ± 0.09	0.74	14	0.42 ± 0.05	0.36 ± 0.07	0.48
PC-B	9	0.60 ± 0.08	0.63 ± 0.14	0.85	15	0.86 ± 0.06	0.49 ± 0.12	0.01
Baseline tension off response*	16	10.52 ± 1.68	14.56 ± 2.90	0.89	23	13.15 ± 1.77	14.59 ± 1.90	0.58
PC-C (off response)	9	1.74 ± 0.11	1.24 ± 0.19	0.02	14	1.46 ± 0.10	1.35 ± 0.15	0.55

See Table 1 for key.

This effect on the "off" response was maximal at lower levels of stimulation (ie, mean ± SEM 6.64 ± 0.61 g decreased to 4.72 ± 0.62 g at 70 V and 2 Hz; 11.00 ± 0.98 g decreased to 6.78 ± 0.79 g at 70V and 4 Hz). In high-tone muscle strips, ATR increased the contractile "off" response to 30 V EFS (Table 3). Conversely to the "on" response alterations, adrenergic blockade did not have an effect on the "off" response at 70 V, but at 30 V, it increased the "off" response in low-tone specimens and again decreased it in high-tone specimens when used in the presence of ATR (Table 4).

Tetrodotoxin induced a significant increase in the contractile "on" response of circularly-oriented tissues at low tone exposed to lower levels of stimulation at 30 and 70 V EFS (mean ± SEM, 0.85 ± 0.31 g for control tissues increased to 1.44 ± 0.38 g at 70 V and 2 Hz; 1.50 ± 0.48 g increased to 2.70 ± 0.64 g at 70 V and 4 Hz). Tetrodotoxin decreased the contractile "off" response to 30 and 70 V EFS (Fig 1 and 4). The same response pattern was seen in high-tone tissues (Fig 2 and 3). Compared with low-tone tissues under cholinergic blockade, TTX induced a significant increase in the contractile "on" response and a significant decrease in the contractile "off" response to EFS (Fig 4). Compared with high-tone strips under adrenergic blockade, TTX significantly decreased the contractile "off" response (ratio, mean ± SEM, 1.57 ± 0.19 decreased to 1.06 ± 0.09 with TTX). Compared with combined adrenergic and cholinergic receptor blockade on high-tone strips, TTX significantly increased the contractile "on" response (Fig 3).

Discussion

Depolarization of myenteric neurons in jejunal smooth muscle strips of horses by use of EFS revealed indirect evidence that ACH and NE can be released from the myenteric plexus. As proof of the hypothesis that ACH acts as an excitatory neurotransmitter in the jejunum of horses, ATR blocked at least a portion of most of the contractile responses to EFS. In other preparations, blockade of adrenergic transmission altered the muscle response to EFS, resulting in relaxation or contraction. We also found evidence for NANC contractile and NANC relaxant neurotransmission. However, we detected significant differences in the response to EFS between the muscle layers and between stimulation parameters.

The longitudinal muscle contractions appeared to be strongly influenced by ACH release from enteric neurons. Atropine inhibited contractile muscle responses under all stimulation conditions, with the exception of high-tone longitudinal muscle subjected to 30 V EFS (Table 1). Tetrodotoxin had effects similar to those of ATR, including the latter finding. Lack of TTX sensitivity could imply that muscle, rather than nerve, was being stimulated. However, other investigators have documented that TTX insensitivity does not necessarily rule out neural involvement in modulating contractility.[20,21] Lack of response to TTX in this circumstance is more likely to result from release of a combination of excitatory and inhibitory neurotransmitters, which led to opposing muscle contraction and relaxation. Similarly, effects of ATR may be masked by release of other neurotransmitters. In preliminary studies performed in the presence of a substance P antagonist, pretreatment with ATR had a significant effect on the muscle response to 30 V EFS (ie, at 8 Hz, ATR decreased the response from 5.66 ± 0.85 g to 2.82 ± 0.73 g; $P < 0.01$). This suggests that cholinergic neurotransmitters are released but masked by release of substance P. Higher intensity stimulation appeared to result in greater cholinergic neurotransmission, perhaps by increasing number of neurons firing, neuronal firing rate, or type of neurotransmitters being released.[22,23,c]

In circularly oriented muscle strips at low tone, the response to ATR was opposite the response to TTX, suggesting that relaxant neurotransmission predominates in these strips. In particular, ATR had minimal effect on the response of muscle strips at low tone to 30 V EFS, consistent with the minimal contractile activity associated with this type of stimulation (Table 3). In general, activity was not observed during application of EFS to circularly oriented tissues at low tone unless high amounts of stimulation were applied, in which case the muscle contracted. The same pattern also was observed by Rakestraw et al[24] in their study of jejunal circular muscle of horses. It appears that release of an inhibitory neurotransmitter maintains a low level of tone to the circular muscle under normal conditions, but higher intensity stimulation can cause circular muscle contraction. This pattern resembles the activity of the circular muscle in the preparatory phase of the peristaltic reflex; the circular muscle typically is relaxed maximally at low filling pressures while the longitudinal muscle contracts. However, when the rate of filling pressure is excessive, the circular muscle also may contract.[20] When detectable, the contractile activ-

ity of ACH on circular muscle appeared to be maximal at low stimulation frequencies. This is comparable to its role in stretch-induced peristalsis in humans in whom cholinergic neurotransmission is predominant at low-level radial stretch, whereas substance P neurotransmission predominates at higher levels of stretch.[25]

Surprisingly, in high-tone circularly oriented muscle strips, ATR caused an increase in the contractile response to EFS (Table 3), which suggests that ACH can act to relax circular smooth muscle when the muscle is at a higher level of tone. This relaxant effect of ACH, as opposed to the contractile effect observed in longitudinal muscle under the same conditions, may explain part of the greater relaxation response observed in high-tone circular muscle during EFS and may reflect differences in neuronal populations or specific receptors between the muscle layers. It is also likely that this pattern of muscle response (relaxation followed by an "off" contraction) is occurring to some degree in studies done at low tone of circular muscle; however, detection of an initial muscle relaxation in those preparations would be more difficult. To induce muscle relaxation, ACH may evoke release of an inhibitory neurotransmitter, inhibit release or effect of an excitatory neurotransmitter, or relax the smooth muscle directly. Results of preliminary studies in our laboratory suggest that ACH may stimulate release of **nitric oxide** (NE), as observed in rabbit blood vessels by Furchgott and Zawadzki.[26] In dogs, ACH has been documented to inhibit cholinergic neurons by acting on presynaptic muscarinic receptors.[27] Fox et al[27] suggested that it may be a natural feedback inhibitory brake to limit contractile responses and prevent or reduce maximal contractions. Similarly, ACH is believed to be involved in the intestino-intestinal inhibitory reflexes of dogs, wherein strong spasmodic contractions in the small intestine initiate a reflexive inhibition of motility proximal and distal to the contraction.[28]

In all situations, the "off" response was inhibited significantly by ATR, indicating a consistent cholinergic component. Physiologically, the aftercontraction may be the means of ensuring return of tone to the intestinal smooth muscle after the relaxation required to allow movement of the food bolus, may reflect the ascending or descending contraction pathways of the peristaltic reflex, or may be part of the mechanism involved in mixing food.[29-31]

Intrinsic adrenergic activity was expected to be minimal in this preparation on the basis of in vivo studies performed in clinically normal horses.[1] However, α-adrenergic receptor blockade with YOH or PTL increased portions of the contractile response to 30 V EFS in longitudinal and circular muscle strips (particularly at 2 to 4 Hz), suggesting that EFS stimulated release of an adrenergic agonist, likely NE, from enteric neurons (Tables 2 and 4). The effects of the adrenergic receptor antagonists were only significant in the presence of ATR. The implication is that a low level of NE-induced relaxation is masked easily by excitatory cholinergic neural depolarization and could explain the lack of detection of adrenergic activity in other studies in horses.

Effects of adrenergic blockade appeared weaker in muscle subjected to higher intensity stimulation, and any effects of NE may have been masked by the release of other neurotransmitters. However, in longitudinal muscle at low tone and in high-tone circular muscle, adrenergic receptor blockade at 70 V EFS inhibited the contractile "on" response, suggesting a contractile response of the muscle after release of NE (Tables 2 and 4). Muscle contraction in response to NE or an α_2-adrenergic agonist has been documented in horses,[11,14,15,32] and muscle contraction in response to NE is present in sphincter areas of the GI tract in many species.[33]

Intrinsic NE release in the jejunum of horses may have a primary role in maintaining inhibitory tone to the intestine, similar to that in other species but at a low level, or myenteric plexus release of NE may serve to relax the intestine after a contraction.[33] In addition to relaxing the GI tract, NE also may have a role in motility regulation by inducing additional muscle contraction in high-tone circular muscle or after high-intensity stimulation to longitudinal muscle. It is possible that these differences between muscle layers and stimulation parameters may correspond to different portions of the peristaltic reflex or may assist in regulating segmentation or mixing of food.[29]

In an attempt to detect NANC neurotransmitter release within the jejunum of horses, we compared the response to EFS in the presence of TTX with that to EFS in the presence of adrenergic and cholinergic receptor blockade. In the longitudinal muscle, TTX induced more significant decreases in the EFS contractile responses than did ATR, particularly during the "off" response (Fig 3). This suggests presence of excitatory non-cholinergic neurotransmission in the longitudinal muscle. In the circular muscle, TTX induced more significant decreases in the contractile "off" response than did ATR (Fig 3) or adrenergic receptor blockade, and it induced more significant increases in the contractile "on" response than did adrenergic receptor blockade (Fig 4). It is possible that TTX blocked more adrenergic or cholinergic transmission than did the specific antagonists; however, these concentrations worked well to suppress the activity of high concentrations of ACH and NE that were used in a previous study in horses.[11] Hence, these findings support the hypothesis that NANC excitatory and inhibitory neurotransmitters are involved in the response of the circular muscle to neuronal depolarization.

The model used in this study worked well to enable separation of the effects of various neurotransmitters in the GI tract of horses. In vitro studies are limited by the inability to monitor intersegmental coordination of gastrointestinal motility and by the inability to directly correlate findings to gastrointestinal activity in the living animal. Additionally, directionality of the responses is lost with the simultaneous release of neurotransmitters involved in local motility as well as in ascending and descending reflexes. The release of neurotransmitters from separate reflexes also may lead to summation responses that are exaggerated or muted. However, in vitro studies often provide the easiest means of analyzing the effects of mechanical alterations or of individual drugs or drug combinations in a controlled setting and may be the only method of

dissecting out the complex interactions between neurotransmitters.[34] Additionally, evaluating drug responses of various muscle layers and under various stimulus conditions may improve our ability to correlate in vitro results with physiologic and pathologic responses.

In conclusion, we found evidence for release of ACH and NE from the myenteric plexus of the jejunum in horses. Acetylcholine appears to be principally a contractile neurotransmitter, but it may cause relaxation of the intestine. On the other hand, although it appears that NE typically induces relaxation after its release, it also may be able to contract the intestine in horses even at nonsphincter sites. Combined adrenergic and cholinergic blockade also revealed the likely involvement of nonadrenergic-noncholinergic neurotransmitters in the jejunum. Furthermore, the type of response to neuronal depolarization was dependent on smooth muscle orientation, level of stimulation, and underlying muscle tone. Inappropriate or incomplete conclusions may be drawn when the function of a single muscle is analyzed, when only maximal EFS is used, or when studies are performed only on muscle strips at one level of tone.

[a]Model S-88, Grass Products, Astro-Med, Inc, West Warwick, RI.

[b]Malone ED. Neural control of equine intestinal motility. PhD Thesis, Department of Clinical and Population Sciences, University of Minnesota, St Paul, Minn, 1998.

[c]Helmy-Elkholy A. Non-adrenergic, non-cholinergic neurons in opossum esophagus. MS Thesis, Department of Neuroscience, McMaster University, Hamilton, Ontario, Canada, 1983.

References

1. Gerring EEL, Hunt JM. Pathophysiology of equine postoperative ileus: effect of adrenergic blockade, parasympathetic stimulation and metoclopramide in an experimental model. *Equine Vet J* 1986;18:249–255.

2. Adams SB. Recognition and management of ileus. *Vet Clin North Am Equine Pract* 1988;4:91–104.

3. Cohen ND, Faber NA, Brumbaugh GW. Use of bethanechol and metoclopramide in horses with duodenitis-proximal jejunitis: 13 cases (1982–1993). *J Equine Vet Sci* 1995;15:492–494.

4. Grider JR. Interplay of VIP and nitric oxide in regulation of the descending relaxation phase of peristalsis. *Am J Physiol* 1993;264:334–340.

5. Sellers AF, Lowe JE, Rendano VT, et al. The reservoir function of the equine cecum and ventral large colon—its relation to chronic non-surgical obstructive disease with colic. *Cornell Vet* 1982;72:233–241.

6. Adams SB, Lamar CH, Masty J. Motility of the distal portion of the jejunum and pelvic flexure in ponies: effects of six drugs. *Am J Vet Res* 1984;45:795–799.

7. Hunt JM, Gerring EL. Effects of autonomic agonists on equine gastrointestinal electromechanical activity, in *Proceedings*. Equine Colic Res 2nd Symp 1986;210–213.

8. Roberts MC, Argenzio A. Effects of amitraz, several opiate derivatives and anticholinergic agents on intestinal transit in ponies. *Equine Vet J* 1986;18:256–260.

9. Ruckebusch Y, Roger T. Prokinetic effects of cisapride, naloxone and parasympathetic stimulation at the equine ileo-caecocolonic junction. *J Vet Pharmacol Ther* 1988;11:322–329.

10. Ringger NC, Lester GD, Neuwirth L, et al. Effect of bethanechol or erythromycin on gastric emptying in horses. *Am J Vet Res* 1996;57:1771–1775.

11. Malone ED, Brown DR, Trent AM, et al. Influence of adrenergic and cholinergic mediators on the equine jejunum in vitro. *Am J Vet Res* 1996;57:884–890.

12. Lester GD, Merritt AM, Neuwirth L, et al. Effect of α_2-adrenergic, cholinergic, and nonsteroidal anti-inflammatory drugs on myoelectric activity of ileum, cecum, and right ventral colon and on cecal emptying of radiolabeled markers in clinically normal ponies. *Am J Vet Res* 1998;59:320–327.

13. Roger T, Ruckebusch Y. Colonic α_2-adrenoceptor-mediated responses in the pony. *J Vet Pharmacol Ther* 1987;10:310–318.

14. Ruckebusch M, Grivel M-L, Fargeas M-J. Sur l'action paradoxale de l'adrenaline au niveau de l'intestin grele chez le cheval. *Arch Int Phramacodyn* 1971;194:387–402.

15. Stick JA, Chou CC, Derksen FJ, et al. Effects of xylazine on equine intestinal vascular resistance, motility, compliance and oxygen consumption. *Am J Vet Res* 1987;48:198–203.

16. Murray A, Cottrell DF, Woodman MP. Cholinergic activity of intestinal muscle *in vitro* taken from horses with and without equine grass sickness. *Vet Res Commun* 1994;18:199–207.

17. Griffiths IR, Kyriakides E, Smith S, et al. Immunocytochemical and lectin histochemical study of neuronal lesions in autonomic ganglia of horses with grass sickness. *Equine Vet J* 1993; 25:446–452.

18. Hodson NP, Wright JA, Hunt J. The sympatho-adrenal system and plasma levels of adrenocorticotrophic hormone, cortisol and catecholamines in equine grass sickness. *Vet Rec* 1986;118:148–150.

19. Eades SC, Moore JN. Blockade of endotoxin-induced cecal hypoperfusion and ileus with an α_2 antagonist in horses. *Am J Vet Res* 1993;54:586–590.

20. Waterman SA, Costa M, Tonini M. Accommodation mediated by enteric inhibitory reflexes in the isolated guinea-pig small intestine. *J Physiol (London)* 1994;474:539–546.

21. Holman ME. Excitation of nerves. In: Daniel EE, Paton DM, eds. *Methods in pharmacology*. Vol 3. New York: Plenum Press, 1975; 299–311.

22. Guyton AC. Neuronal mechanisms and circuits for processing information. In: Guyton AC, ed. *Textbook of medical physiology*. 7th ed. Philadelphia: WB Saunders Co, 1986;562–571.

23. Hoyle CHV, Burnstock G. Criteria for defining enteric neurotransmitters. In: Gaginella TS, ed. *Methods in gastrointestinal pharmacology*. New York: CRC Press, 1996;123–140.

24. Rakestraw PC, Snyder JR, Woliner MJ, et al. Involvement of nitric oxide in inhibitory neuromuscular transmission in equine jejunum. *Am J Vet Res* 1996;57:1206–1213.

25. Grider JR. Identification of neurotransmitters regulating intestinal peristaltic reflex in humans. *Gastroenterology* 1989;97: 1414–1419.

26. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980;288:373–376.

27. Fox JET, Daniel EE, MacDonald TJ, et al. Evidence for a muscarinic inhibitory brake activated by peptides in the canine small intestine. In: Roman C, ed. *International symposium on gastrointestinal motility*. Lancaster: MTP Press Ltd, 1984;327–333.

28. Frantzides CT, Sarna SK, Matsumoto T, et al. An intrinsic neural pathway for long intestino-intestinal inhibitory reflexes. *Gastroenterology* 1987;92:594–603.

29. Guilford WG. The enteric nervous system: function, dysfunction, and pharmacological manipulation. *Semin Vet Med Surg (SA)* 1990;5:46–56.

30. Goyal RK, Hirano I. The enteric nervous system. *N Engl J Med* 1996;334:1106–1115.

31. Barthó L, Holzer P. Search for a physiological role of substance P in gastrointestinal motility. *Neuroscience* 1985;16:1–32.

32. Sellers AF, Lowe JE, Cummings JF. Trials of serotonin, substance P and α_2-adrenergic receptor effects on the equine large colon. *Cornell Vet* 1985;75:319–323.

33. Gillis RA, Souza JD, Hicks KA, et al. Inhibitory control of proximal colonic motility by the sympathetic nervous system. *Am J Physiol* 1981;253:531–539.

34. Percy WH. In vitro techniques for the study of gastrointestinal motility. In: Gaginella TS, ed. *Methods in gastrointestinal pharmacology*. New York: CRC Press, 1996;189–224.