Expression of the ether-a-go-go (ERG) potassium channel in smooth muscle of the equine gastrointestinal tract and influence on activity of jejunal smooth muscle

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Objective—To determine whether ether-a-go-go (ERG) potassium channels are expressed in equine gastrointestinal smooth muscle, whether ERG channel antagonists affect jejunal muscle contraction in vitro, and whether plasma cisapride concentrations in horses administered treatment for postoperative ileus (POI) are consistent with ERG channels as drug targets.

Sample Population—Samples of intestinal smooth muscle obtained from 8 horses free of gastrointestinal tract disease and plasma samples obtained from 3 horses administered cisapride for treatment of POI.

Procedure—Membranes were prepared from the seromuscular layer of the duodenum, jejunum, ileum, cecum, large colon, and small colon. Immunoblotting was used to identify the ERG channel protein. Isolated jejunal muscle strips were used for isometric stress response to ERG channel blockers that included E-4031, MK-499, clofilium, and cisapride. Plasma concentrations of cisapride were determined in 3 horses administered cisapride for treatment of POI after small intestinal surgery.

Results—Immunoblotting identified ERG protein in all analyzed segments of the intestinal tract in all horses. The selective ERG antagonist E-4031 caused a concentration-dependent increase in jejunal contraction. Clofilium, MK-499, and cisapride also increased jejunal contraction at concentrations consistent with ERG channel block; effects of E-4031 and cisapride were not additive. Peak plasma cisapride concentrations in treated horses were consistent with ERG block as a mechanism of drug action.

Conclusions and Clinical Relevance—The ERG potassium channels modulate motility of intestinal muscles in horses and may be a target for drugs. This finding may influence development of new prokinetic agents and impact treatment of horses with POI.

Postoperative ileus (POI) represents an important problem in the management of horses that have undergone surgery of the gastrointestinal (GI) tract. Costs associated with POI can be substantial in terms of client financial responsibility as well as morbidity and mortality rates of horses. In 1 review, approximately 85 to 90% of deaths of horses in a hospital during the short-term postoperative period were attributable to POI. The magnitude of this clinical problem has heightened interest in the development of pharmacologic protocols that promote GI motility in horses. Unfortunately, specific knowledge about the efficacy and potential adverse effects of prokinetic drugs in horses has developed slowly because of the limited evaluation of drugs in horses with experimentally induced ileus, small number of prospective clinical studies, and gradual accumulation of empirical data.

The practical use of prokinetic agents in horses has been limited because of adverse reactions or incomplete efficacy. For example, metoclopramide has been associated with severe undesirable CNS effects and failure to coordinate motility in the distal aspect of the small intestine. Clinical use of erythromycin as a prokinetic agent has been limited by concerns about induction of severe diarrhea as a result of the drug’s antimicrobial activity and by conflicting reports about its efficacy. Cardiac toxicosis attributable to cisapride has been associated with an unacceptable incidence of events associated with cardiac toxicosis in humans.

Cardiac toxicosis attributable to cisapride has been associated with antagonism of potassium channels formed by subunits from the ether-a-go-go (ERG) family of channel proteins. In another study conducted by our laboratory group, we reported that these ERG potassium channels are expressed in the heart of horses and that cisapride interferes with normal repo-
larization of equine cardiac muscle. Thus, it would be beneficial to find an effective prokinetic agent devoid of ERG blocking activity for use in horses. The feasibility of this goal depends entirely on whether antagonism of ERG potassium channels in the GI tract is important for the prokinetic effects of cisapride. Currently, we are not aware of data that would support or refute this possibility.

Cisapride is a serotonin (5-HT) receptor agonist. Its prokinetic effects in various species and various segments of the intestinal tract are reportedly mediated by at least two 5-HT-receptor-mediated pathways, 1 involving release of acetylcholine from nerve terminals and 1 involving direct effects on smooth muscle. It has been suggested that the stimulatory effects of cisapride on equine jejunal smooth muscle are mediated primarily through non-cholinergic mechanisms, at least in part by 5-HT2 receptors; however, these findings do not preclude a role for potassium channels as additional targets for drugs.

Results of studies conducted in a number of laboratories provide direct and indirect support for the hypothesis that blockade of ERG channels may contribute to the prokinetic effects of cisapride in horses. For example, ERG potassium channels and currents expressed in opossum esophagus and rat stomach determine resting membrane potentials, control smooth muscle excitability, and serve as molecular targets for cisapride. Furthermore, domperidone, another prokinetic drug with documented efficacy for use in horses with POI, can block ERG channels at clinically relevant concentrations of the drug. The documented ability of cisapride to alleviate endotoxin-induced delay of gastric emptying also is consistent with a mechanism of action that involves ERG blockade and smooth muscle depolarization. Reactive oxygen species increase outward potassium currents through ERG channels, which would favor increased (more negative) resting membrane potentials and relaxation (rather than contraction) of GI smooth muscle.

We are not currently aware of any information about expression of potassium channels at any location in the intestines of horses. The ability of potassium-channel antagonists administered at clinically relevant concentrations to increase contractions of GI smooth muscle has not been evaluated. The study reported here was designed to address these important questions. Equine intestinal membrane fractions were probed for expression of ERG channel proteins by use of western immunoblot analysis. The effects of specific ERG potassium-channel antagonists on jejunal smooth muscle contraction were determined in vitro. To define the range of cisapride concentrations that have clinical relevance, plasma concentrations of cisapride were measured in horses that had been administered the drug as a treatment for POI.

Collection of samples—Immediately after each horse was euthanized, full-thickness segments of the duodenum, jejunum, ileum, cecum, large colon, and small colon were obtained via an incision made in the right flank. The tunica serosa and tunica muscularis were separated from the submucosa and mucosa by sharp dissection. Approximately 2 to 3 g of seromuscular layer from each portion of bowel were washed in PBS solution prepared from a commercially available 10X stock solution supplemented with protease inhibitor cocktail (1.500 dilution); specimens were snap-frozen in liquid nitrogen and stored at −70°C for future use in western immunoblot analysis. Additional portions of jejunum were harvested from 4 horses and transported to our laboratory for use in an in vitro investigation of jejunal smooth muscle activity.

Preparation of membranes—Frozen specimens of GI tissues obtained from 5 horses were cut into small pieces and then homogenized in Tris-EDTA buffer (20mM Tris-HCl and 1mM EDTA [pH, 7.4]). Nuclei and debris were pelleted by centrifugation at 12,000 × g for 10 minutes. The procedure was repeated, and supernatants were pooled. Membrane fractions were removed from the pooled supernatant by centrifugation at 40,000 × g for 90 minutes. Membrane pellets were solubilized in buffer (PBS solution with 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and a dilution [1:500] of protease inhibitor cocktail) and stored in aliquots at −70°C. All buffers and reagents used during the experiments.

In immunoblots, lysates of HEK-293 cells transfected with ERG protein and untransfected HEK-293 cells were included as positive and negative controls, respectively.

In vitro investigation of jejunal smooth muscle activity—Portions of the mid-jejunum obtained from 4 euthanized horses were used to investigate smooth muscle activity. Ingesta were removed by washing the lumen with modified Krebs-Ringer’s buffer (KRB) solution (110mM NaCl, 4.6mM KCl, 2.5mM CaCl2, 24.8 mM NaHCO3, 1.2mM KH2PO4, 1.2mM MgSO4, and 5.6mM glucose [pH, 7.4]). Specimens were transported to the laboratory in chilled, oxygenated KRB solution. Full-thickness muscle strips (2 × 10 mm) were cut parallel to the circular muscle fibers, and then the mucosa and submucosa were removed by sharp dissection. The resultant GI smooth muscle strips (8 to 16 strips/horse) were suspended separately in tissue baths containing 20 mL of KRB solution maintained at 37°C and continuously aerated with 95% O2-5% CO2. One end of each muscle strip was secured to an isometric force transducer. Muscle strips were allowed to equilibrate for 60 minutes before 1 g of tension was applied to stimulate spontaneous contractions. Tissue baths were flushed with fresh KRB solution containing atropine (2mM) and guanethidine (2mM) to inhibit cholinergic and adrenergic neurotransmission; these drugs were included in subsequent solutions used during the experiments.

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**Materials and Methods**

Sample population—Muscle samples were obtained from the GI tract of horses donated to the veterinary medical teaching hospitals at Kansas State University and Texas A&M University. Horses were euthanized after incurable musculoskeletal or neurologic disease was diagnosed. Horses were euthanized by administration of sodium pentobarbital (85 to 110 mg/kg, IV). The study was approved by the respective institutional animal care and use committees.

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After regular phasic contractile activity was established, strips were exposed to KRB solution containing various concentrations of a potassium channel blocking drug or its vehicle (control solution). Isometric responses were recorded by use of a force transducer interfaced with an 8-channel chart recorder and computer-based data acquisition and analysis systems. Responses were recorded for a minimum of 4 minutes after addition of drug or vehicle. Muscle strips exposed to vehicle alone were included in all experiments as control samples for the effects of solvent and time.

To generate cumulative concentration-response relationships for cisapride (1 to 30 \( \mu \text{M} \)) and E-4031 (1nM to 5 \( \mu \text{M} \)), muscle strips were exposed to progressively increasing concentrations of drug. Muscle strips were exposed to clofilium tosylate (1 \( \mu \text{M} \)), MK-499 (5 \( \mu \text{M} \)), cisapride (5 \( \mu \text{M} \)), E-4031 (5 \( \mu \text{M} \)), or a mixture of cisapride and E-4031 (5 \( \mu \text{M} \) each) in separate sets of experiments. Dilutions of cisapride in KRB solution were made from a 10mM stock solution of cisapride dissolved in dimethyl sulfoxide. Dilutions of other drugs in KRB solution were made by use of stock solution of 1mM E-4031, 1mM MK-499, and 3mM clofilium dissolved in double-distilled H2O.

**Plasma concentrations of cisapride**—Blood samples were obtained from horses (3 clinical cases) that were administered cisapride (0.1 mg/kg, IV, q 6 h) for the treatment of POI that developed after small intestinal surgery. A diagnosis of POI was made in each horse when there was nasogastric reflux of greater than 1 L/h when the nasogastric tube was in place, decreased sounds in the GI tract, heart rate within the reference range, and normal hydration status. Cisapride solution (4 mg/mL) was prepared for IV administration by dissolution of drug in tartaric acid followed by sonication and sterilization by filtration. Blood samples were collected by jugular venipuncture 0 (prior to administration), 15, 30, 45, 60, 120, and 480 minutes after the administration of cisapride. Plasma was obtained after centrifugation at 4°C and stored at −70°C. Samples were shipped by overnight courier on dry ice to another researcher’s laboratory.

**Data analysis**—The response of jejunal smooth muscle to various concentrations of drug or vehicle was assessed by plasma concentrations of cisapride—Blood samples

![Figure 1](image1.png)

![Figure 2](image2.png)

**Figure 1**—Western immunoblot of immunoreactive ether-a-go-go (ERG) protein at approximately 145 and 100 kD in equine intestinal muscle membrane fractions (approx 10 \( \mu \text{g/lane} \)) from the duodenum (D), jejunum (J), ileum (I), cecum (C), ventral colon (Vc), and small colon (Sc). Lysates of HEK-293 cells transfected with ERG protein and untransfected HEK-293 cells are included as positive-control (+) and negative-control (−) samples. Molecular weight markers (in kilodaltons) are in the left-hand column. Data shown are representative of results obtained with tissues from each of 5 horses.

**Figure 2**—Stimulatory effects of the ERG channel antagonist E-4031 (panels A to C) and MK-499 (panel D) on contractile force of isolated equine jejunal smooth muscle. Panel A — Concentration-dependent effects of E-4031 on the area under the curve (AUC) for isometric force vs time curve associated with the 180-second interval between 60 and 240 seconds after addition of the drug to the muscle bath (AUC\text{60-240}). Each point represents the mean ± SEM value for 10 muscle strips obtained from 4 horses. *Value differs significantly (\( P \leq 0.05 \)) from values of lower concentrations. Panel B — Adjusted concentration-response curve for data in panel A. Line represents the best-fit dose-response function as calculated by use of the Hill equation. Panel C — Response of isolated jejunal smooth muscle exposed throughout the same time period to increasing concentrations of E-4031 (solid square) or vehicle (solid circle). Each point represents the mean ± SEM value for 4 muscle strips obtained from 1 horse. Panel D — Contractile force (tension) as a function of time for 1 muscle strip before and after exposure to MK-499. Introduction of the MK-499 (5 \( \times \) \( 10^{-5} \text{M} \)) to the tissue bath is indicated (arrow).
comparison of the area under the curve (AUC) for the isometric response as a function of time, specifically the AUC associated with the 180-second interval between 60 and 240 seconds after addition of drug to the muscle bath (AUC\textsubscript{60-240}). Graphic presentation and statistical comparison of data were accomplished by use of commercially available software. Data were reported as mean ± SEM. Treatment effects were assessed by use of a paired t-test or repeated-measures ANOVA, when appropriate. Differences were considered significant at \( P ≤ 0.05 \). When the value for the overall F test was significant, pairwise comparisons of means were performed by use of the least significant difference method.

**Results**

**Western immunoblot analysis**—An equine homologue of ERG was identified by immunoblotting with an antibody directed against a C-terminal epitope. Two bands corresponding to molecular masses of ERG protein in cardiac tissues of horses\textsuperscript{19} were observed at 145 and 100 kD in all analyzed segments of the intestinal tract (Fig 1). Similar results were obtained for intestinal smooth muscle membranes collected from each of the 5 horses.

**Jejunal smooth muscle activity**—Addition of E-4031, a specific antagonist of ERG potassium channels,\textsuperscript{31} significantly increased the AUC\textsubscript{60-240} of drug-treated jejunal muscle strips (10 muscle strips obtained from 4 horses) in a concentration-dependent fashion (Fig 2). Relationship between the adjusted response (response reported as a fraction of the maximal observed response to drug) and logarithmic concentration of E-4031 was sigmoidal, as expected for a physiologic effect mediated by a saturable drug-ion channel interaction. Enhancement of AUC\textsubscript{60-240} was not observed for control preparations similarly exposed to vehicle alone (4 muscle strips from 1 horse). Comparable stimulatory effects on jejunal smooth muscle were observed for other potassium-channel antagonists. Addition of MK-499, another specific antagonist of ERG channels,\textsuperscript{34} increased phasic contractions in 3 preparations in which the drug's effects were examined. Clofilium (1\(\mu\)M), an antagonist of ERG as well as other voltage-gated potassium channels\textsuperscript{35,36} also increased the values for AUC\textsubscript{60-240} (Fig 3).

As expected, the mean ± SEM AUC\textsubscript{60-240} of jejunal smooth muscle strips treated with 1\(\mu\)M cisapride (54.5 ± 15.8 g · s) was significantly greater than that of strips treated with only vehicle (32.4 ± 5.2 g · s). Higher concentrations of cisapride did not result in any additional increases in the observed AUC\textsubscript{60-240} (mean values of 55.1 ± 16.4 and 54.3 ± 16.1 g · s for treatment with 3 and 10\(\mu\)M cisapride, respectively). Effects of maximally effective concentrations of E-4031 and cisapride on AUC\textsubscript{60-240} were not additive (Fig 4).

**Plasma concentrations of cisapride**—Plasma concentration-time curves were determined for the 3 horses administered cisapride for treatment of POI (Fig 5). The 3 horses had persistent nasogastric reflux of 3 to 4
L/h and a heart rate within the reference range 72 hours after surgery, which was prior to administration of cisapride. All 3 horses were discharged after resolution of clinical signs.

**Discussion**

Results reported here provide biochemical and pharmacologic evidence for the expression of ERG potassium channels in smooth muscle of the GI tract of horses, and analysis suggests that these voltage-gated ion channels may be a target for prokinetic drugs. Furthermore, the data document that the plasma concentrations of cisapride in horses treated because of POI are consistent with the potential for substantial drug blockade of ERG potassium channels in cardiac and intestinal muscle.

Western immunoblot analysis of ERG revealed that an N-linked glycosylated mature form of the channel protein (approx 145 kD) and an alternatively spliced variant (approx 100 kD) were expressed throughout the equine GI tract. Data from native cardiac tissue and heterologous expression systems strongly suggest that the N-glycosylated form of ERG (approx 145 kD) is efficiently trafficked to the membrane and available to form functional channels. Thus, our immunoblotting data are consistent with detection of ERG potassium channels in intestinal smooth muscle of horses. Both forms of ERG have been detected in cardiac tissues of horses, whereas only the higher molecular weight form was found in rat stomach.

The selective ERG potassium-channel antagonists, E-4031 and MK-499, and the less selective potassium-channel antagonist, clofilium, increased contractile force in isolated jejunal muscle strips. Furthermore, the effects of E-4031 and cisapride were not additive. These data document that ERG potassium channels modulate intestinal motility in horses and are consistent with reports that ERG channels are involved in control of resting potential and contractile activity in esophageal tissues of opossums and stomach smooth muscle of rats. Micromolar concentrations of E-4031 and cisapride induced depolarization and also elicited spontaneous phasic contractions in vitro in isolated esophageal muscle strips of opossums; the amplitude and character of the E-4031-induced contractions were similar to those seen for jejunal smooth muscle of the horses reported here.

Cisapride has direct stimulatory effects on isolated feline and equine intestinal smooth muscle. The drug's prokinetic action on feline colon is dependent on a dihydropyridine-sensitive pathway. This finding is compatible with cisapride-induced antagonism of ERG potassium channels, because blockade of outward potassium current would cause myocyte depolarization and thereby enhance calcium entry through voltage-gated, L-type calcium channels. Direct in vitro stimulatory effects of cisapride on equine jejunum have been attributed to 5-HT3 receptors, because the 5-HT3 receptor antagonist, ketanserin, attenuated the drug's prokinetic effects. However, ketanserin also has direct effects on ERG and other potassium channels. Thus, interpreted in the context of those published data, analysis of the data reported here strongly suggests that intestinal ERG potassium channels in horses represent molecular targets for prokinetic drugs and that the same may hold true for other companion animals.

Plasma concentrations of cisapride were measured in 3 horses that were treated with the drug because of POI associated with small intestinal surgery. Two of these horses had peak plasma concentrations (Cmax) of cisapride within the range (97 to 231 ng/mL) reported for healthy horses administered an identical dose of cisapride by the IV route. However, the third horse had a substantially higher Cmax of 550 ng/mL. The median inhibitory concentration for cisapride blockade of ERG channels in transfected mammalian cells ranges from 6 to 45 nM. The tissue-to-plasma ratio of cisapride is approximately 5:1 in rabbits, although similar measurements have not been made in horses. Thus, our data confirm that plasma and tissue concentrations of cisapride in horses can be comparable to the tissue bath concentrations used in the study reported here and in another in vitro study of motility of intestinal muscles in horses. In another study conducted by our laboratory group, we documented that 1 µM cisapride delays repolarization of action potentials and induces early after-depolarizations in equine cardiac muscle. When considered together, findings from that study and the study reported here also suggest that horses given cisapride to prevent or treat POI are at risk for cisapride-induced cardiac arrhythmias.

Improving the survival rate of horses that undergo surgery to correct GI tract conditions is an important clinical goal of veterinarians. Rational use of prokinetic agents to stimulate normal GI motility is an important aspect of this overall objective, because most of the acute deaths of horses in veterinary hospitals after intestinal surgery are associated with POI. The failure to identify important molecular targets for prokinetic drugs in the equine intestine limits severely the ability of veterinarians to extrapolate results obtained in humans or other species to horses. Assuming, as our data suggest, drug interactions with ERG potassium channels in the equine intestine are important for the prokinetic effects of cisapride, current efforts to develop and market noncardioversion versions of cisapride (ie, substituted benza-mides that retain cisapride interactions with 5-HT receptors but not with ERG potassium channels) may yield drugs that do not have substantial efficacy for horses with POI. Under such circumstances, effective prokinetic agents for horses would be more likely to result from discovery of drugs designed to target ERG potassium channels in intestinal smooth muscle selectively over ERG potassium channels in cardiac or other tissues.

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1GIBCO, Invitrogen, Carlsbad, Calif.
2P-8340, Sigma Chemical Co, St Louis, Mo.
3155942, ICN, Aurora, Ohio.
4Micro BCA protein assay, Pierce Biochemical, Rockford, Ill.
5cTryptophan, Sigma Chemical Co, St Louis, Mo.
6155942, ICN, Aurora, Ohio.
7G-8520, Sigma Chemical Co, St Louis, Mo.
8Model 70 polygraph, National Instruments, Austin, Tex.
9WINDAQ, Dataq Instruments Inc, Akron, Ohio.
10Cisapride, Research Diagnostics Inc, Flanders, NJ.
11E4031, Wako Pure Chemicals Industries Ltd, Osaka, Japan.
12Clofilium tosylate, Sigma-Aldrich Corp, St Louis, Mo.
13MK499, Merck Research Labs, West Point, Pa.
References