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Expression and function of 5-HT₇ receptors in smooth muscle preparations from equine duodenum, ileum, and pelvic flexure

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Abstract

In horses, gastrointestinal (GI) disorders occur frequently and cause a considerable demand for efficient medication. 5-Hydroxytryptamine receptors (5-HT) have been reported to be involved in GI tract motility and thus, are potential targets for treating functional bowel disorders. Our studies extend current knowledge on the 5-HT₇ receptor in equine duodenum, ileum and pelvic flexure by studying its expression throughout the intestine and its role in modulating contractility *in vitro* by immunofluorescence and organ bath experiments, respectively.

5-HT₇ immunoreactivity was demonstrated in both smooth muscle layers, particularly in the circular one, and within the myenteric plexus. Interstitial cells of Cajal (ICC), identified by c-Kit labeling, show a staining pattern similar to that of 5-HT₇ immunoreactivity.

The selective 5-HT₇ receptor antagonist SB-269970 increased the amplitude of contractions in spontaneous contracting specimens of the ileum and in electrical field-stimulated specimens of the pelvic flexure concentration-dependently.

Our *in vitro* experiments suggest an involvement of the 5-HT₇ receptor subtype in contractility of equine intestine. While the 5-HT₇ receptor has been established to be constitutively active and inhibits smooth muscle contractility, our experiments demonstrate an increase in contractility by the 5-HT₇ receptor ligand SB-269970, suggesting it exerting inverse agonist properties.

Keywords

5-HT₇ receptor; Interstitial cells of Cajal; c-Kit; Equine; Intestine; Motility

1. Introduction

Gastrointestinal (GI) disorders are among the ten most frequently observed health problems in horses holding a considerable risk of mortality. The authors state that GI disorders pose a case fatality risk of about 5–8% (Cohen et al., 2004; Kaneene et al., 1997).

Moreover, surgical procedures in horses require anesthesia for several hours and often lead to functional inhibition of propulsive bowel motility, designated as postoperative ileus (POI)

(Cohen et al., 2004) The incidence of POI in horses was reported to be about 15% (Mair and Smith, 2005). Today, a wide range of drugs including local anesthetics, macrolide antimicrobials, cholinergic agonists, dopamine antagonists, and drugs acting on serotonin receptor subtypes are in use for POI therapy in horses (Smith et al., 2005). However, none of these compounds is satisfactory in terms of efficacy and safety. Cisapride, a 5-HT₄ (5-hydroxytryptamine receptor 4) agonist with 5-HT₃ and dopamine receptor 2 (D₂) antagonistic properties that was frequently used in equine POI therapy (Van Hoogmoed et al., 2004) in recent years, is no longer available on the market due to well-known cardiac safety issues in humans. Cisapride was reported to cause prolongation of the QT interval (Paakkari, 2002) associated with torsades de pointes and sudden cardiac death in humans (Smith and Gallagher, 1980).

Consequently, there is further need for research to identify receptors involved in equine GI motility and to investigate the effect of compounds targeting these receptors. There is evidence that serotonin receptor subtypes are involved in GI tract motility (Mohammad-Zadeh et al., 2008). Serotonin is known to markedly influence bowel function by activating at least five receptor types (5-HT₁₋₄ and 5-HT₇). Among 5-HT receptors, 5-HT₃, an ionotropic receptor, forming an ion channel pore that is opened or closed in the direct response to 5-HT, and 5-HT₄, a metabotropic receptor, which is indirectly linked with ion-channels through G proteins, have been most extensively studied in gastroenterology. Drugs targeting 5-HT receptors have proven effective to modulate *in vitro* GI motility of many species including humans (Gershon, 2004), dogs, guinea-pigs (Grider et al., 1998; Tonini et al., 2005), rats (Grider et al., 1998), cats (Washabau and Sammarco, 1996) and horses (Delesalle et al., 2006; Nieto et al., 2000; Weiss et al., 2002). Seven families of serotonin receptors, designated 5-HT₁ to 5-HT₇ (Hoyer et al., 2002), are currently recognized and subtypes have been described for several of them. 5-HT receptors mainly associated with mediating relaxation of GI tract include 5-HT₁ (Coulie et al., 1997) and 5-HT₇ (Tuladhar et al., 2003) receptors, whereas 5-HT₂ (Briejer et al., 1997), 5-HT₃ (Gershon, 2000) and 5-HT₄ (Gershon, 2000) receptors have been reported to stimulate GI tract smooth muscle contractility. Most of the seven families of 5-HT receptor subtypes known today have been identified in the GI tract of mammals (Glatzle et al., 2002; Johnson and Heinemann, 1995; Makimoto et al., 2002; Meylan et al., 2004; Poole et al., 2006; Zhao et al., 2003).

Recently, much effort has been devoted to study the role of 5-HT₄ receptors in GI motility in equine medicine. *In vivo* studies were performed in horses, demonstrating a prokinetic effect of the 5-HT₄ (partial) agonists tegaserod (Lippold et al., 2004) and mosapride (Sasaki et al., 2005). In contrast, only a few functional studies on equine 5-HT₇ receptors are available.

5-HT₇ receptors were reported to be expressed by neurons in addition to smooth muscle cells in guinea-pig ileum (Tonini et al., 2005). Although effects of compounds targeting 5-HT₇ receptors in the CNS have been described, little information on the effect of 5-HT₇ receptor agonists or antagonists on GI motility is available in the literature (Thomas and Hagan, 2004). However, there is evidence that they are involved in smooth muscle relaxation, including the GI tract. (Thomas and Hagan, 2004). The selective 5-HT₇ antagonist SB-269970 has been reported to antagonize the relaxant response of agonists targeting 5-HT₇ receptors in pulmonary arteries in weaned pigs (Jahnichen et al., 2005), and in canine proximal stomach longitudinal muscle strips (Janssen et al., 2002). It has been hypothesized that 5-HT₇ receptors are involved in the modulation of circular muscle tone in guinea-pig ileum (Tonini et al., 2005). However, in a comprehensive study by Delesalle et al. (2006), a selective 5-HT₇ agonist was shown to exert no effect on contractility in equine longitudinal jejunum *in vitro* (Delesalle et al., 2006).

The aim of our *in vitro* study was to investigate the expression of 5-HT₇ receptor subtypes along the equine intestinal tract and to evaluate to which extent this receptor could contribute

to smooth muscle contractility. Three different locations of the GI tract were chosen, two from the small intestine, due to its frequent involvement in equine GI tract disorders. Because the impact of 5-HT₇ receptor modulation in equine jejunum was already evaluated (Delesalle et al., 2006) *in vitro*, our study was aimed to investigate effects of the selective 5-HT₇ receptor antagonist SB-269970 in duodenum and ileum. In addition, pelvic flexure, a hairpin turn in the large colon, which is predisposed to obstruction, was chosen as a representative sample of the large bowel because of its important functional role in regulating colonic aboral and retropropulsive transit of digesta through its motility pacemaker activity (Lopes and Pfeiffer, 2000).

It is established that specialized cells known as interstitial cells of Cajal (ICC) found within the *tunica muscularis* of the GI tract are important for the integrity of GI motility (Ward and Sanders, 2006). In particular, ICC serve as electrical pacemakers, provide pathways for the active propagation of slow waves, are mediators of enteric motor neurotransmission and play a role in afferent neural signaling (Ward and Sanders, 2006). Hudson et al. (1999) demonstrated the occurrence of ICC in the circular muscle layer and the myenteric region in equine GI tract by using anti-c-Kit IgG as marker for ICC.

In a double-labeling immunofluorescence approach expression and possible co-localization of 5-HT₇ receptors and c-Kit, a tyrosine kinase receptor present on ICCs was investigated.

In a functional study, we evaluated the effect of 5-HT₇ receptors on contractility by using the selective 5-HT₇ antagonist SB-269970 in organ bath experiments.

2. Materials and methods

2.1. Tissue samples and preparation

Equine intestinal tissue was obtained from 29 horses of different breeds and sexes from local slaughterhouses. The horses, aged between one and 30 years, had no previous history of GI tract disease. Intestinal specimens of duodenum, ileum and pelvic flexure were collected immediately after slaughtering. Specimens from the duodenum were taken 20 cm aboral to the pylorus. Ileal samples were taken at the proximal insertion site of the plica ileocaecalis (ileocaecal fold), and specimens from pelvic flexure were taken at the position, where all taeniae are discontinued. The bowel content was immediately removed by rinsing the specimens with cooled (5 °C) PBS (PBS Tablets, Calbiochem, (10 mM phosphate buffer, pH 7.4, 140 mM NaCl and 3 mM KCl)) or Krebs–Henseleit-(KH) solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, and 11 mM glucose). Freshly prepared PBS or KH solution was used for immersion of the tissues during the 20 min transport to the laboratory. Intestinal pieces of about 3 × 5 cm in size were then promptly abscised from the mucosa and kept in cooled PBS for the immunofluorescence experiments or KH solution aerated with carbogen (95% O₂, 5% CO₂) until their use in the organ bath experiments.

2.2. Immunofluorescence

We used data from the first assembly of the horse genome sequence (<http://www.tiho-hannover.de/einricht/zucht/hgp/index.htm>) and equine 5-HT₇ receptor gene sequence available in the GenBank database (AY927689) to select the most adequate antibody for detecting 5-HT₇ receptors in this species.

For double-labeling immunofluorescence, intestinal specimens from three horses were obtained as described in Section 2.1. The specimens were transported in cooled PBS solution. Strips of about 15 × 5 mm were prepared, pinned onto polystyrene plates and flash-frozen in liquid nitrogen. Cryostat sections of 5 µm thickness were cut either parallel to the longitudinal or to the circular muscle layer. All steps in the immunofluorescence staining procedure were

performed at room temperature. Briefly, the slides were fixed in 2% paraformaldehyde in PBS for 30 min. After 20 min of treatment with 0.2 M glycine in PBS, followed by 20 min incubation in 0.3% Triton X-100, the slides were incubated with 5% normal donkey serum in 3% BSA supplemented with streptavidin solution (Streptavidin/Biotin Blocking Kit SP-2002, Vector Laboratories) for 1 h according to the manufacturers' instructions. A mixture of affinity-purified goat polyclonal anti-SCF receptor/c-Kit antibody (R&D Systems, 4 ng/ μ l) and affinity-purified rabbit polyclonal anti-5-HT₇ receptor antibody (Novus Biologicals, 10 ng/ μ l) supplemented with biotin solution (Streptavidin/Biotin Blocking Kit SP-2002, Vector Laboratories) was used according to the manufacturers' specifications. Incubation with primary antibodies was performed for 12 h. Affinity-purified, Cy2-conjugated donkey antigoat IgG (1:200), affinity-purified, biotin-SP-conjugated donkey anti-rabbit IgG (1:3000) and Cy3-conjugated streptavidin (1:3000) (all from Jackson ImmunoResearch, (West Grove, PA, USA) were used for detection. All immunolabeling reagents were diluted in DAKO antibody diluent (DAKO Corp., Carpinteria, CA, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, MO, USA).

For the double-labeling experiments, specificity controls were performed by omitting primary antibodies and using an irrelevant rabbit primary antibody directed against the calcitonin G-protein coupled receptor (10 ng/ μ l, Abcam, Cambridge, UK), respectively.

All preparations were washed extensively in PBS after immunostaining and were subsequently mounted using Fluoprep (Biomérieux, Marcy l'Etoile, France). Immunofluorescence was observed with a Zeiss Axioskop (Carl-Zeiss AG, Feldbach, Switzerland) equipped with an AxioCam HR digital camera (Carl-Zeiss AG), Feldbach, Switzerland). All experimental and control images of the immunofluorescence data were collected at identical imaging settings. Contrast were adjusted identically for experimental and corresponding control photos using ImageJ software (<http://rsb.info.nih.gov/ij>).

2.3. Experimental protocols

For organ bath experiments, intestinal specimens were obtained as described in Section 2.1. using KH solution as transport solution. Stripes of 10 × 3 mm were dissected in parallel to the longitudinal muscle layer. The smooth muscle preparations were suspended in individual Schuler tissue baths (Hugo Sachs Electronics) filled with oxygenated KH solution at 37 °C. Preparations were randomly allocated to the individual organ bath chambers. The distal end of each muscle strip was clamped to a hook and the proximal part was attached to an isometric force transducer (Type 351, Hugo Sachs Electronics) as described previously (Kenny et al., 2002; Weiss et al., 2002). Smooth muscle contractility was recorded on a personal computer and analyzed using the Power Lab data acquisition system (ADInstruments). Each preparation was used only once to avoid tachyphylaxis.

2.3.1. Spontaneous contractions—Smooth muscle preparations were placed in the organ baths and after 10 min, the tissue was challenged with 1×10^{-6} M carbachol (Sigma Chemical Co., St. Louis, MO, USA) to check the viability of preparations. Subsequently, the organ baths were flushed three times and the initial muscle tension was routinely preset to 1 g. Smooth muscle preparations were allowed to equilibrate for 80 min prior to starting the experiment. The organ bath chambers were flushed with KH solution after 30 min and the tension was readjusted after 60 min. Specimens featuring regular spontaneous activity, including constant frequency, amplitude, and basal tone of contraction for at least 10 min prior to the start of the experiment, were chosen as subjects for testing. Subsequently, cumulative concentration–response curves were generated using the SB-269970 5-HT₇ antagonist (Sigma Chemical Co., St. Louis, MO, USA) in specimens of duodenum, ileum and pelvic flexure at increasing organ

bath concentrations in logarithmic steps ranging from 10^{-10} to 10^{-5} M at 5 min intervals. Solvent controls were conducted in the same way.

2.3.2. Electrical field stimulation—Initial resting tension was routinely set to 1 g. During a 30 min equilibration period, the KH solution was exchanged every 15 min and the tension was readjusted. Thereafter, electrical field stimulation (EFS) was initiated. The stimulation pattern consisted of three trains of 5 s at an interval of 20 s and impulses of 125 mA with a duration of 5 ms at a frequency of 50 Hz using a Grass S 88 stimulator (Grass Instruments). The parameters were chosen to evoke contractions being regular in pattern and in which the induced frequency was adjusted to resemble the frequency of spontaneous contractions. After 45 min, the specimens were checked for activity (amplitude of contractions of phasic contractions) and, where required, the current intensity was increased gradually every 5 min until contractility was observed. This submaximal amplitude of contractions allows to register an increase or a decrease in the amplitude of contractions. Only specimens contracting regularly at least 10 min prior to the designated experimental start were included in the study. Concentration–response curves were constructed for the 5-HT₇ antagonist SB-269970 and its matching solvent (H₂O) by stepwise addition of SB-269970 in concentrations ranging from 10^{-10} to 10^{-6} M in half-logarithmic steps every 5 min.

2.4. Data analysis and statistics

The following contractility parameters were analysed: basal tone (BT) and maximal amplitude of contractions ($A_{\max}$). In experiments using spontaneous contractions, the frequency, defined by number of contractions per min (cpm), was analysed in addition to BT and $A_{\max}$. All contractility parameters were recorded at 5 min intervals using PowerLab and ChartTM software (ADInstruments). Recording of contractility parameters started 5 min prior to the cumulative addition of compounds (predrug) and ceased 5 min after the last addition of compound. All data are expressed relative to the predrug period.

The data obtained from the cumulative concentration-response effect were analyzed for any experimental protocol for each intestinal location using the Friedman test (SYSTAT, SPSS, Inc, Chicago, IL, USA). Subsequently, significant effects over time were subject to compound comparisons using ANOVA for repeated measures followed by the Bonferroni multiple comparison test using the NCSS software (Number Cruncher Statistical Systems, Kaysville, UT, USA). Values are presented as mean and standard error of the mean (SEM); P values < 0.05 were considered statistically significant. Concentration–response curves were calculated using the Hill equation and estimation by use of the least squares method (MAT-LAB Simulation Software, The MathWorks, Natick, MA, USA). The underlying equation for the Hill function is:

$$\text{Response} = V_{\max} \cdot C^{\alpha} \cdot (C^{\alpha} + K^{\alpha})^{-1}$$

where $V_{\max}$ is the maximal attainable response, C is the compound concentration, K stands for the half-effective concentration (EC_{50}), and the exponent α describes the shape of the function. Statistical significance of comparisons made on the basis of this model was determined using the likelihood ratio statistic which yields a chisquared test. Standard deviations presented for parameters in the Hill model are based on the Cramer-Rao statistic (Portier et al., 1993). The results were expressed as $V_{\max}$ and EC_{50} of $A_{\max}$.

3. Results

3.1. Immunofluorescence

In all locations examined, strong 5-HT₇ receptor immunoreactivity was detected in the region of the myenteric plexus (Fig. 1). The intensity of staining was not conspicuously different between small and large intestine. 5-HT₇ immunoreactivity was present in some elongated cells in circular and longitudinal muscle layers, as shown by the example of pelvic flexure in Fig. 2. The stained cells appear to have their long axes oriented parallel to the surrounding muscle fibres. Coincident localization of 5-HT₇ receptor and c-Kit immunoreactivity was found in all locations investigated in most of the cells in the myenteric plexus (Fig. 1), and in some elongated cells in both muscle layers (Fig. 2). Because c-Kit is a specific marker of ICC, labeled cells will be referred to as ICC. Distinct cells within the longitudinal muscle layer were immunoreactive for 5-HT₇ receptor but not for c-Kit (Fig. 2a), demonstrating the occurrence of 5-HT₇ receptors on non-ICC cells.

The negative controls were devoid of specific immunolabeling but show some unspecific staining (general background) (Fig. 1 and Fig. 2).

3.2. Spontaneous contractions

Bowel specimens exhibiting regular contractile activity at least 10 min prior to the start of the experiment were the only ones considered in the present study. Thus, only 67% (18 out of 27) of duodenal preparations, 57% of ileal preparations (20 out of 35) and 51% (22 out of 43) of pelvic flexure specimens were included in the analysis. At the beginning of the analysis, the mean frequency of spontaneous contractions was 10.43 (SEM 2.19), 6.31 (SEM 1.02) or 3.94 (SEM 2.02) cpm with a mean $A_{\max}$ of 1.21 (SEM 0.31), 0.83 (SEM 0.21) or 5.67 (SEM 1.93) g, and a basal tone of 0.86 (SEM 0.11), 1.17 (SEM 0.12) or 0.99 (SEM 0.20) g for duodenal, ileal and pelvic flexure preparations, respectively.

The 5-HT₇ antagonist SB-269970, had no effect on BT in all locations of the equine intestine investigated.

The addition of SB-269970 caused an increase in $A_{\max}$ in specimens from the duodenum, ileum and pelvic flexure (Fig. 3) with the effect being statistically significant only for ileal specimens when compared to solvent controls (Fig. 3b).

No significant differences in EC₅₀ values were found for specimens from different locations. $V_{\max}$ values were estimated to be significantly higher in specimens from equine duodenum ($V_{\max} = 0.86$ g) compared to pelvic flexure ($V_{\max} = 0.56$ g; $P < 0.05$) for the amplitude of contractions (Table 1). No difference in $V_{\max}$ was seen for the amplitude of contractions when ileal specimens were compared to preparations from duodenum or pelvic flexure (Table 1).

A concentration-dependent effect of SB-269970 on the frequency of contractions could not be determined in specimens from all intestinal locations.

3.3. Electrical field stimulation

At the beginning of the analysis, the mean frequency of EFS-induced contractions was 4.66 (SEM 0.66), 4.24 (SEM 0.69) or 3.42 (SEM 0.28) cpm with a mean $A_{\max}$ of 2.52 (SEM 0.71), 1.32 (SEM 0.36) or 1.74 (SEM 0.52) g, and a basal tone of 1.18 (SEM 0.17), 1.28 (SEM 0.25) or 1.06 (SEM 0.14) g for duodenal, ileal and pelvic flexure preparations, respectively.

In specimens of all locations investigated, SB-269970 did not change the BT over time when compared to solvent controls.

In preparations from ileum and pelvic flexure, SB-269970 triggered a significant increase in $A_{\max}$, as shown in Fig. 4b and c. In preparations from pelvic flexure, this effect was also significantly different from matching solvent controls (Fig. 4c).

In duodenal samples, no significant effect in $A_{\max}$ over time was observed (Fig. 4a and c). This effect was also significantly different from matching solvent controls.

No difference in EC_{50} values was estimated for the amplitude of contractions when the effect of SB-269970 was compared among locations of the equine intestine investigated (Table 1). The maximal obtainable response for $A_{\max}$ was more pronounced in specimens from pelvic flexure when compared with preparations from equine ileum and duodenum with a statistically significant effect. The maximal obtained response for $A_{\max}$ was estimated to be higher in ileum than in duodenum. The data are presented in Table 1.

4. Discussion

To our knowledge, this study is the first report on the presence of 5-HT₇ receptors in equine intestine. We provide evidence for widespread expression of 5-HT₇ receptors and c-Kit in equine duodenum, ileum and pelvic flexure. As revealed in single- and double-labeling immunofluorescence experiments, anti-c-Kit antibodies result in a similar staining pattern as anti-5-HT₇ antibodies, suggesting a potential co-localization. The observed overlap in staining pattern observed suggests a close vicinity of 5-HT₇ receptors and c-Kit that might indicate a functional relationship of the two. C-Kit is a tyrosine kinase receptor, which is expressed on the surface of ICC and therewith facilitates reliable identification of ICC (Huizinga et al., 1995). ICC are considered to be the most important cells related to GI contractility due to their role as electrical pacemakers: ICC generate spontaneous electrical slow waves and conduct them to smooth muscle cells, where a contraction is initiated. Our results are consistent with findings demonstrating that ICC are found in the equine intestine particularly in the circular muscle layer and in the region of the myenteric plexus. We could confirm the existence of ICC in all intestinal locations investigated. Cells in the region of the myenteric plexus and elongated cells in both muscle layers, particularly in the circular muscle, stained positive when tested with antibodies against c-Kit, indicating evidence for ICC. Similar to the findings reported by Hudson et al. (1999), sectioning of tissues in different planes showed that ICC tend to have their long axes orientated parallel to muscle fibres. Hudson et al. (1999) observed a higher concentration of c-Kit-like immunoreactivity in the myenteric plexus of the small intestine and in the circular muscle layer of the large intestine. In contrast, we found that the distribution pattern of ICC appeared to remain constant throughout the GI tract with prominent staining in the myenteric plexus region in both, small and large intestine, and moderate staining of scattered cells/cell groups within both muscle layers. However, a quantitative analysis was not performed in our study.

Co-localization of the ICC marker c-Kit with other 5-HT receptor subtypes in the GI tract has been reported in other species. In ileal tissues of rat (Glatzle et al., 2002) and mice (Liu et al., 2005), co-localization of 5-HT₃ or 5-HT₄ receptors and ICC has been shown. Tonini et al. (2005) demonstrated the expression of the 5-HT₇ receptor by myenteric neurons in addition to smooth muscle cells in guinea-pig ileum, which is in agreement with our findings.

The incidence of spontaneous contractile activity differed among locations within the equine intestine: a higher contractile activity was observed in muscle preparations originating from duodenum and ileum (67% resp. 57% activity) as compared to pelvic flexure (51% activity). Various reasons can be considered for that. A higher contractile activity in an area is not necessarily correlated with a higher occurrence of pacemaker cells. ICC in different parts of GI tract may have different properties, which may affect frequency or amplitude of

contractions. Moreover, other inputs (e.g. enteric- and autonomic nervous system) might also affect contractility.

The frequency of spontaneous contractions declined gradually from 10 cpm in the duodenum and 6 cpm in the ileum to 4 cpm in the pelvic flexure. This corresponds to the frequency of slow waves observed in the human GI tract with 11–12 cpm in the duodenum, 8 cpm in the ileum and 3–6 cpm in the large bowel (Lin et al., 2006; Sullivan et al., 1977). Hudson et al. (1999) demonstrated highest densities of c-Kit-positive ICC in equine ileum, pelvic flexure and body of caecum, but not in duodenum. Hence, the reported (Hudson et al., 1999) distribution pattern of ICC along the equine bowel does not correlate with the variability in the incidence of spontaneous contractions in longitudinal smooth muscle preparations. Other explanatory approaches, whether based on morphological or molecular differences of anatomical locations, are therefore required to explain this observation.

The enterochromaffin cells of the enteric mucosa are the main source of 5-HT in the body. These cells respond to mechanical and chemical stimulation by releasing 5-HT that, in turn, stimulates neurons, initiating peristaltic activity (Grider et al., 1998). There is some evidence that 5-HT₇ receptors are involved in GI motility (Thomas and Hagan, 2004). The present findings strengthen the idea that 5-HT₇ receptors play an important role in intestinal peristalsis and may act as target of endogenous 5-HT released by neuronal and non-neuronal cells such as enterochromaffin cells. We used cumulative concentration of the selective 5-HT₇ antagonist SB-269970 to evaluate the effects of this substance on spontaneous contractions and on EFS-induced contractions.

Interestingly, in our study, SB-269970 caused an increase in the amplitude of spontaneous and EFS-induced contractions. However, this increase in spontaneous contractions was only significant for ileal specimens, but not for duodenal and pelvic flexure specimens. In EFS-induced contractions the increase was significant for preparations of pelvic flexure, but not for small intestinal specimens. Examining contraction parameters before the experimental start of specimens from the pelvic flexure more closely, $A_{\max}$ was less pronounced in EFS-induced contractions at the beginning of the experiment when compared to spontaneous contractions. A submaximal amplitude of contractions facilitates the observation of a significant increase in $A_{\max}$ in tissues from pelvic flexure in EFS-induced contractions when compared to spontaneous contractions.

In the literature, controversial findings on the effect of SB-269970 in the GI tract can be found: *in vitro*, SB-269970 exhibited no effect on 5-HT-induced contractility in equine jejunal specimens (Delesalle et al., 2006), but an increase in contractility was reported in electrically-induced contractions of dog longitudinal antrum (Prins et al., 2001). These heterogeneous findings may indicate different responses to SB-269970 due to a difference in distribution of 5-HT₇ receptor subtypes between species and between anatomic sites. Different methodologies used in the studies could also explain the different outcomes. In contrast to our study, Delesalle et al. (2006) investigated the effect of SB-269970 on 5-HT-induced basal tonic contractions, whereas phasic contractility was not determined. Our goal was to evaluate the potential of SB-269970 by measuring its effects on BT, $A_{\max}$ and frequency of spontaneous contractions, and on BT and $A_{\max}$ of EFS-mediated contractions, in which the induced frequency was adjusted to resemble the frequency of spontaneous contractions. SB-269970 did not change the basal tone in a significant manner, which is in agreement with the findings reported by Delesalle et al. (2006). The increase in amplitude of contractions mediated by SB-269970 in equine ileum, can be explained by its properties as an inverse agonist. Inverse agonists, previously known as classical antagonists, bind to the same binding-site as an agonist for that receptor but exert the opposite pharmacological effect. There is previous evidence from a study using chinese hamster ovary cells expressing human recombinant 5-HT₇ receptors and

HEK-293F cells expressing rat and human 5-HT₇ receptors for SB-269970 being an inverse agonist (Mahe et al., 2004; Romero et al., 2006; Sprouse et al., 2004). A 'quasi-full inverse agonist' is defined as a ligand that can cause a response being considerably higher than that elicited by a partial inverse agonist, but not as high as that elicited by a full inverse agonist. The effect of an inverse agonist depends on the constitutive activity of the receptor.

According to the experimental results obtained with equine jejunum 5-HT mediates its effect through non-neurogenic, noncholinergic pathways (Delesalle et al., 2006). This suggests the effect of SB-269970 to be exclusively mediated by muscular 5-HT₇ receptors. However, as observed in guinea-pig (Tonini et al., 2005) and in this study in equine tissue, 5-HT₇ receptors were found to be highly expressed in the region of the myenteric plexus. A more detailed investigation of the function of myenteric 5-HT₇ receptors is therefore needed.

5. Conclusions

In conclusion, demonstration of 5-HT₇-immunoreactive cells in myenteric plexus and smooth muscle layers provides evidence for the existence of 5-HT₇ receptors in the equine duodenum, ileum and pelvic flexure, and for their expression on ICC or at least in close proximity to ICC. These findings are supported by functional *in vitro* experiments, demonstrating an increase in contractility of spontaneous contractions in equine ileum and of EFS-induced contractions in pelvic flexure mediated by the 5-HT₇ receptor ligand SB-269970.

According to current knowledge on the 5-HT₇ receptor as a mediator of relaxation, our experiments are indicative of inverse agonist properties of SB-269970.

Our data suggests 5-HT₇ receptors in horses are involved in modulation of GI tract contractility, which makes them possible targets for drug development. However, the inconsistent *in vitro* effects of 5-HT₇ receptor ligand SB-269970 observed in our study as compared to previous reports warrant closer examination with respect to the anatomical location and the experimental protocols used. Furthermore, *in vivo* studies are necessary to demonstrate whether 5-HT₇ antagonists like SB-269970 are beneficial for treatment of GI motility disorders, such as POI.

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Fig. 1.

5-HT₇ receptor and c-Kit immunoreactivity were localized in the equine duodenum (a), ileum (b) and pelvic flexure (c) by immunofluorescence staining on cryostat sections. Intestinal specimens were double-labeled with anti-5-HT₇ receptor and anti-c-Kit IgGs (a1–a4, b1–b4, c1–c4). Primary antibodies were omitted in matching controls (a5–a8, b5–b8, c5–c8). Detection was performed using Cy2-(green, c-Kit: a1, b1, c1, resp. control: a5, b5, c5) and Cy3-(red, 5-HT₇ receptor: a2, b2, c2, resp. control: a6, b6, c6) labeled secondary antibodies. Nuclei were stained with DAPI (blue, a3, b3, c3, resp. a7, b7, c7). Merged images of c-Kit- and 5-HT₇ receptor immunoreactivity and DAPI (a4, b4, c4) or matching controls (a8, b8, c8). MP, myenteric plexus; CM, circular muscle layer; LM, longitudinal muscle. Scale bar = 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 2.

5-HT₇ receptor and c-Kit immunoreactivity were localized in the longitudinal (LM, a) and circular (CM, b) muscle layers of the equine pelvic flexure with anti-5-HT₇ receptor and anti-c-Kit IgGs (a1–a4, b1–b4). Primary antibodies were omitted in matching controls (a5–a8, b5–b8). Detection was performed using Cy2-(green, c-Kit: a1, b1, resp. control: a5, b5) and Cy3-(red, 5-HT₇ receptor: a2, b2, resp. control: a6, b6) labeled detection reagents. Nuclei were stained with DAPI (blue, a3, b3 resp. a7, b7). Merged images of c-Kit- and 5-HT₇ receptor immunoreactivity and DAPI (a4, b4) and matching controls (a8, b8). Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 3. Concentration-dependent effects of the 5-HT₇ antagonist SB-269970 on maximal amplitude (A_{max}) of spontaneous contractions in longitudinal specimens of equine duodenum (a), ileum (b), and pelvic flexure (c). Data are given as mean and SEM. Significant differences in the response of SB-269970 compared to solvent (H₂O) are indicated by + ($P < 0.05$). Significant effects over time are denoted by \$.

Fig. 4. Concentration-dependent effects of the 5-HT₇ antagonist SB-269970 on the maximal amplitude (A_{max}) of electrical field-stimulated contractile activity in longitudinal specimens of equine duodenum (a), ileum (b), and pelvic flexure (c). Data are given as mean and SEM. Significant differences in the response of SB-269970 compared to solvent (H₂O) are given as + ($P < 0.05$). Significant effects over time are denoted by \$.

Table 1
Concentration-dependent response of the 5-HT₇ antagonist SB-269970 on $V_{\max}$ and EC₅₀ values of the maximal amplitude of contractions in longitudinal specimens of equine intestine.

Specimen	Protocol	<i>n</i>	$V_{\max}$	Lower CL	Upper CL	EC ₅₀ (M)
Duodenum	SC	9	0.86 ^a	3.59×10^{-5}	4.62×10^4	1.0×10^{-10}
Duodenum	EFS	8	0.27 ^b	0.03	2.42	1.0×10^{-10}
Ileum	SC	8	0.83	2.66×10^{-5}	2.68×10^4	1.31×10^{-10}
Ileum	EFS	11	0.39 ^b	0.08	2.00	1.15×10^{-9}
PF	SC	8	0.56 ^a	3.41×10^{-7}	9.14×10^5	1.0×10^{-10}
PF	EFS	8	0.76 ^b	0.24	2.39	4.41×10^{-9}

Abbreviations: PF = pelvic flexure, SC = spontaneous contractions, EFS = electrical field stimulation, *n* = number of experiments, $V_{\max}$ = maximal attainable effect, CL = 95% confidence interval, EC₅₀ = half-effective concentration. $V_{\max}$ values are given in g; EC₅₀ values are given in M. Within columns, values with the same superscript are significantly different from each other ($P < 0.05$).