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Contractile effects of 5-hydroxytryptamine and 5-carboxamidotryptamine in the equine jejunum

*,[1]Cathérine Delesalle, [1]Piet Deprez, [2]Jan A.J. Schuurkes & [3]Romain A. Lefebvre

[1]Department of Large Animal Internal Medicine, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; [2]Johnson & Johnson Pharmaceutical Research and Development, a division of Janssen Pharmaceutical N.V., Turnhoutseweg 30, B-2340 Beerse, Belgium and [3]Heymans Institute of Pharmacology, Ghent University, De Pintelaan 185, Ghent, Belgium

1 The use of human prokinetic drugs in colic horses leads to inconsistent results. This might be related to differences in gastrointestinal receptor populations. The motor effects of 5-hydroxytryptamine (5-HT; serotonin) on the equine mid-jejunum were therefore studied. Longitudinal muscle preparations were set up for isotonic measurement.

2 5-HT induced tonic contractions with superimposed phasic activity; these responses were not influenced by tetrodotoxin and atropine, suggesting a non-neurogenic, non-cholinergic pathway.

3 The 5-HT receptor antagonists GR 127935 (5-$HT_{1B,D}$), ketanserin (5-HT_{2A}), SB 204741 (5-HT_{2B}), RS 102221 (5-HT_{2C}), granisetron (5-HT_3), GR 113808 (5-HT_4) and SB 269970 (5-HT_7) had no influence on the 5-HT-induced response; the 5-HT_{1A} receptor antagonists NAN 190 ($pK_b = 8.13 \pm 0.06$) and WAY 100635 ($pK_b = 8.69 \pm 0.07$), and the 5-$HT_{1,2,5,6,7}$ receptor antagonist methysergide concentration-dependently inhibited the 5-HT-induced contractile response.

4 The 5-$HT_{1,7}$ receptor agonist 5-carboxamidotryptamine (5-CT) induced a contractile response similar to that of 5-HT; its effect was not influenced by tetrodotoxin and atropine, and SB 269970, but antagonised by WAY 100635. 8-OHDPAT, buspiron and flesinoxan, which are active at rat and human 5-HT_{1A} receptors, had no contractile influence.

5 These results suggest that the contractile effect of 5-HT in equine jejunal longitudinal muscle is due to interaction with muscular 5-HT receptors, which cannot be characterised between the actually known classes of 5-HT receptors.
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Abbreviations: 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino) tetralin; GABA, gamma-aminobutyric acid; GR 127935, 2-methyl-4-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]amide HCl; GR 113808, [1-[2-[(methylsulphonyl) amino]ethyl]-4-piperidinyl]methyl-1-methyl-1*H*-indole-3-carboxylate; i.v., intravenous; L-NNA, N^G-nitro-L-arginine; NAN-190, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine HCl; RS 102221, 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulphon-amido)phenyl-5-oxopentyl)]-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride; SB204741, *N*-(1-methyl-5-indolyl)-*N*′-(3-methyl-5-isothiazolyl)urea; SB 269970, (*R*)-3-(2-(2-(4-methylpiperidin-1-yl) ethyl)pyrrolidine-1-sulphonyl) phenol; TTX, tetrodotoxin; WAY 100635, *N*-2-4-(2-methoxyphenyl)-1-piperazinylethyl-*N*-(2-pyridinyl)cyclohexane carboxamide trihydro-chloride

Introduction

Postoperative ileus is a notorious complication in horses that is predominantly seen after surgical intervention for small intestinal colic. Ileus in horses is characterised by a loss of adequate and coordinated intestinal motility and propulsion leading to the production of large amounts of gastric reflux and small intestinal distention. This complication is responsible for as many as 86% of equine deaths following abdominal surgery (Roussel *et al*., 2001). The pathogenic mechanisms which have been implicated as possible causes are sympathetic inhibitory reflexes, parasympathetic hypoactivity, dopaminergic hyperactivity and inhibitory mediators of the inflammatory response (Gerring & Hunt, 1986; Morris, 1991).

The goals of postoperative treatment are maintenance of adequate hydration, correction of electrolyte imbalance, pain relief, control of infection and last but not least, restoration of normal intestinal propulsion. The latter however often poses a real therapeutic challenge. The mainstays of currently used prokinetic treatments are extrapolated from human medicine by use of cisapride, metoclopramide, domperidone and erythromycin (Van Hoogmoed *et al*., 2004). Also, postoperative intravenous (i.v.) administration of lidocaine is inspired by human use (Brianceau *et al*., 2002). Up until now, however, application of these prokinetic treatments is invariably associated with inconsistent to poor results in horses with ileus.

An overview of the literature shows the lack of fundamental *in vitro* research on the equine intestine to justify the routine use of these human prokinetic drugs in colic horses. There is insufficient scientific evidence that the already established enteral receptor populations that serve as pharmacological target to induce intestinal propulsion in humans are equally

*Author for correspondence; E-mail: Catherine.Delesalle@UGent.be

important in horses. A possible discrepancy in these receptor populations between humans and horses could partially explain the inconsistent clinical efficacy of human prokinetic agents in equine colic cases.

Recently, increasing scientific interest in the role of serotonin (5-hydroxytryptamin; 5-HT) in human gastrointestinal motility has led to the development of several compounds of potential interest for the treatment of functional gastrointestinal tract disorders. The gastroprokinetic effect of the recently introduced tegaserod in humans is, as for cisapride, related to the activation of 5-HT_4 receptors on cholinergic neurons, facilitating release of the contractile neurotransmitter acetylcholine (Talley, 2001). In healthy horses, tegaserod administered intravenously was shown to accelerate gastrocolonic transit of barium-filled particles given *via* a stomach tube and identified radiographically in the collected faeces; it increased the frequency of defaecation and the gut sounds at the caecal base (Lippold *et al*., 2004). Little information is available on the *in vitro* characterization of the 5-HT receptor population in the equine gut. In equine jejunum circular muscle, Nieto *et al*. (2000) reported that the stimulatory effect of 5-HT was antagonised by a 5-HT_2 and a 5-HT_3 receptor antagonist, but not by a 5-HT_4 receptor antagonist. Both atropine and tetrodotoxin (TTX) had no effect on the 5-HT-induced contractions, which suggests that in this part of the intestine 5-HT mediates its effect through 5-HT_2 and 5-HT_3 receptors, active *via* a non-neurogenic, noncholinergic pathway. This is very peculiar, since up until now a solely neuronal localisation has been ascribed to the 5-HT_3 receptor. The stimulatory effect of cisapride, which was less pronounced than that of 5-HT, was not influenced by atropine plus TTX and was attributed to 5-HT_2 receptor activation, based on the antagonistic effects of the specific 5-HT_2 receptor antagonist ketanserin. Again this observation is surprising, since cisapride has only been characterised as a 5-HT_2 receptor antagonist. For equine ileum and pelvic flexure circular and longitudinal muscle, Weiss *et al*. (2002) reported stimulatory effects of 5-HT that were reduced by 5-HT_4 receptor antagonism, but still more by 5-HT_3 receptor antagonism, so that an interaction with 5-HT_3 and 5-HT_4 receptors was proposed; tegaserod had a stimulatory effect that was less pronounced than that of 5-HT.

The aim of this study was to identify the contractile serotonergic receptor population in the small intestine of the horse, taking into account all serotonergic receptor-type possibilities, and thus not limiting the study to the testing of the presence of 5-HT receptor populations identified in human intestine, being mainly 5-HT_2, 5-HT_3 and 5-HT_4 receptors. The rationale to investigate primarily small intestine is the fact that postoperative ileus is predominantly located in this intestinal segment. The jejunal longitudinal smooth muscle was elected because up until now no 5-HT receptor population characterization has been performed in this muscle layer.

Methods

Tissue collection and smooth muscle strip preparation

The study population was comprised of horses of various breeds and either sex, with an age range of 2–20 years. Ponies, foals and draft horses were excluded from the study.

Segments of the middle part of the equine jejunum were collected at the slaughterhouse, using the ileum as point of orientation. Shortly after stunning, the gastrointestinal tract was removed from the carcasses and a jejunal segment of 20 cm was dissected at a distance of 8 m proximal to the jejunoileal junction. The segments were then rinsed with oxygenated Krebs–Henseleit solution (composition in mM: glucose 11.1, $CaCl_2$ 2.51, $NaHCO_3$ 25, $MgSO_4$ 1.18, KH_2PO_4 1.18, KCl 4.69 and NaCl 118) at 4°C, to remove bowel contents and were subsequently immersed in the same oxygenated solution during transportation to the laboratory.

Within 1 h after tissue collection, the intestinal segments were opened along the mesenteric border and were carefully cleared of mucosa, submucosa and mesenterium. Strips (maximum 32 per horse) of approximately 1.5 cm length and 4–5 mm width were then prepared in the direction of the longitudinal muscle layer and mounted onto tissue holders. These were placed in a set-up of 16 organ baths, containing Krebs–Henseleit solution (20 ml) at 37°C, continuously gassed with 95% O_2 and 5% CO_2. The mechanical activity of the preparations was recorded *via* isotonic transducers (Harvard apparatus) coupled to a 16-channel PowerLab (ADInstruments, Melbourne, Australia), under a load of 2 g. The load of 2 g was determined as optimal by preliminary testing on strips of 10 horses, measuring maximal carbachol-induced contraction under loads ranging from 1 up to 10 g.

A 1-h stabilisation period was allowed before the start of the experiment, during which the organ baths were flushed with Krebs–Henseleit solution at 30 and 60 min. After this period, regular spontaneous activity was observed in all preparations. Subsequently, the tissue was challenged twice with 1 μM carbachol at an interval of 30 min. This induced in all preparations two tonic contractions of similar size, illustrating complete equilibration of the tissue.

Experimental protocols

Preliminary experiments with 5-HT In preliminary experiments, the responses to cumulative administration of 5-HT (0.1 nM to 1 μM) within the same tissue were compared with those to administration of eight increasing concentrations of 5-HT (0.1 nM to 3 μM) in eight parallel jejunal strips of the same horse (one concentration per tissue). This learned that the cumulative concentration–response curve to 5-HT was clearly depressed at the higher concentrations of 5-HT in comparison to the isolated one (see Results), so that only isolated concentration–response curves were obtained in further experiments with 5-HT and other 5-HT receptor agonists. Preliminary experiments also indicated that repeated administration of 0.1 μM 5-HT at 15 min interval (with washout once the contractile response was obtained) led to a decreasing response to 5-HT already at the second administration. When the interval was increased to 30 min, the response to repetitive administration of 0.1 μM 5-HT (up to seven times) remained stable.

Influence of TTX and atropine, N^G-nitro-L-arginine (L-NNA) and 5-HT receptor antagonists on the response to 5-HT TTX (0.3 μM) plus atropine (0.3 μM), and L-NNA (100 μM) were tested *versus* 5-HT as follows. An isolated concentration–response curve to 5-HT was constructed by administering eight increasing concentrations of 5-HT to eight

jejunal strips of a horse (thus each preparation only receiving one concentration of 5-HT), and a parallel curve to 5-HT was obtained after incubation for 20 min with TTX plus atropine, or L-NNA in eight strips of the same horse. A series of 5-HT receptor antagonists was tested *versus* 5-HT in the same way: ketanserin (5-HT_{2A}; 0.3 μM), granisetron (5-HT_3; 0.3 μM); GR 113808 ([1-[2-[(methylsulphonyl)amino]ethyl]-4-piperidinyl]-methyl-1-methyl-1*H*-indole-3-carboxylate, 5-HT_4; 0.1 μM); SB 269970 ((*R*)-3-(2-(2-(4-methylpiperidin-1-yl) ethyl)pyrrolidine-1-sulphonyl) phenol, 5-HT_7; 0.3 μM); methysergide (5-$HT_{1,2,5,6,7}$; 1, 10 and 100 nM), NAN 190 (5-HT_{1A}; 0.1, 0.3 and 1 μM) and WAY 100635 (*N*-2-4-(2-methoxyphenyl)-1-piperazinylethyl-*N*-(2-pyridinyl)cyclohexane carboxamide trihydro-chloride, 5-HT_{1A}; 3, 30 and 300 nM).

TTX (3 μM) and atropine (1 μM) were also tested separately *versus* 1 μM 5-HT. 5-HT was added twice at 30 min interval; 20 min before the second administration, TTX (3 μM) and/or atropine (1 μM) were added to the organ bath; a third tissue was used as a control. The following 5-HT receptor antagonists were also tested in the same way: GR 127935 (2-methyl-4-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]amide HCl, 5-$HT_{1B,D}$; 0.1 μM), ketanserin (5-HT_{2A}, 0.3 μM), SB 204741 (*N*-(1-methyl-5-indolyl)-*N*′-(3-methyl-5-isothiazolyl)urea, 5-HT_{2B}; 0.3 μM), RS 102221 (8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulphon-amido)phenyl-5-oxopentyl)]-1, 3, 8-triazaspiro[4.5]decane-2,4-dione hydrochloride, 5-HT_{2C}; 0.3 μM), granisetron (5-HT_3; 0.3 μM) and GR 113808 (5-HT_4; 0.1 μM).

The above-described experiments showed that WAY 100635, NAN 190 and methysergide were the only 5-HT receptor antagonists, with a clearcut influence on the effect of 5-HT. Therefore, they were also tested in the following way. 5-HT (0.1 μM) was added seven times at 30 min interval with washout after the contractile response was obtained in four tissues of the same horse; 20 min before the second to sixth administration of 5-HT, increasing concentrations of WAY 100635, NAN 190 or methysergide were added; the seventh administration of 5-HT was carried out after washout of the antagonists. The fourth tissue of the same horse was used as a control.

Influence of other 5-HT receptor agonists Isolated concentration–response curves were also constructed for the 5-HT_{1A} receptor agonists flesinoxan, 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino) tetralin) and buspiron, and for 5-carboxamidotryptamine (5-CT; 5-$HT_{1,7}$). TTX (0.3 μM) plus atropine (0.3 μM), SB 269970 (0.3 μM) and WAY 100635 (3, 30 and 300 nM) were tested *versus* isolated concentration–response curves of 5-CT, as described for 5-HT. The influence of TTX (3 μM) and atropine (1 μM) was also tested separately *versus* 1 μM 5-CT, as described *versus* 1 μM 5-HT. The possible antagonistic effect of flesinoxan (0.1 μM), 8-OH-DPAT (0.1 μM) and buspiron (1 μM) *versus* 5-HT was tested by adding 0.1 μM 5-HT twice at 30 min interval; 20 min before the second administration, flesinoxan, 8-OH-DPAT or buspiron was added. The concentrations of flesinoxan, 8-OH-DPAT and buspiron in these experiments were chosen to be at least 100 times higher than their affinity values determined from competition binding with [^{3}H]8-OH-DPAT in CHO cells expressing the human 5-HT_{1A} receptor (Newman-Tancredi *et al*., 2001).

Drugs

The following drugs were used (abbreviations and respective suppliers in parentheses): carbachol (Merck, Germany), 5-hydroxytryptamine (5-HT; Janssen Research foundation, Belgium), atropine sulphate (Merck, Germany), methysergide maleate, ketanserin tartrate, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)-butyl]piperazine HCl (NAN-190), SB204741, GR 113808, GR 127935, L-NNA, granisetron HCl, RS 102221, SB 269970 (Janssen Research foundation, Belgium), TTX (Serva, Germany), 5-CT (Tocris Cookson, UK), 8-OH-DPAT, flesinoxan, buspiron (Janssen Research foundation, Belgium); WAY 100635 (Tocris Cookson, UK). All compounds were dissolved in distilled water, except for NAN 190 that was dissolved in distilled water with 10% cyclodextrin, and SB 204741 that was dissolved in distilled water with 20% cyclodextrin. The solvents had no effect on the muscle strips *per se* and did not affect the agonist and antagonist concentration–response curves. All stock solutions were prepared freshly on the day of the experiment and dilutions were prepared using distilled water.

Data analysis

Data collection was performed using Chart for Windows (v4.12, ADInstruments, Oxfordshire, UK).

The amplitude of contractions induced by 5-HT and 5-HT receptor agonists is expressed as % of the second carbachol-induced contraction. In the experiments where increasing concentrations of the antagonists methysergide, WAY 100635 and NAN 190 were tested *versus* 0.1 μM 5-HT, the amplitude of contractions is normalised by expressing them as % of the blanco 5-HT-induced contraction before administration of these antagonists, used as reference.

Concentration–response curves to 5-HT and other agonists were individually fitted to the Hill equation using a computerised iterative nonlinear curve fitting procedure, obtaining curve parameter estimates for upper asymptote E_{max}, midpoint location pEC_{50} and Hill slope n_H. Curve parameters in the presence of an antagonist were compared to those in its absence by unpaired *t*-test, accepting competitive antagonism when the pEC_{50} was significantly decreased but E_{max} and slope were not significantly altered. In case of competitive antagonism, the pK_b of the antagonist was calculated according to log $K_b = \log B - \log(DR-1)$. When the influence of a single concentration of antagonist was tested *versus* a single concentration of 5-HT (1 μM), within the same tissue, the responses to 5-HT in the absence and presence of the antagonist were compared by a paired *t*-test. When several concentrations of a 5-HT receptor antagonist (NAN 190, WAY 100635) were tested in one single strip, *versus* a fixed dose of 5-HT (0.1 μ), K_b values of the antagonists were calculated using the logistic function described by Lazareno & Birdsall (1993), which represents a modification of the Cheng–Prusoff equation for analysing antagonist inhibition curves in functional experiments:

$$K_b = \frac{IC_{50'}}{\frac{[A_f]}{EC_{50'}} - 1}$$

where K_b is the antagonist dissociation constant and $[A_f]$ is the fixed agonist concentration (in this case 5-HT 0.1 μM). For reasons of accuracy and convenience, when using this method,

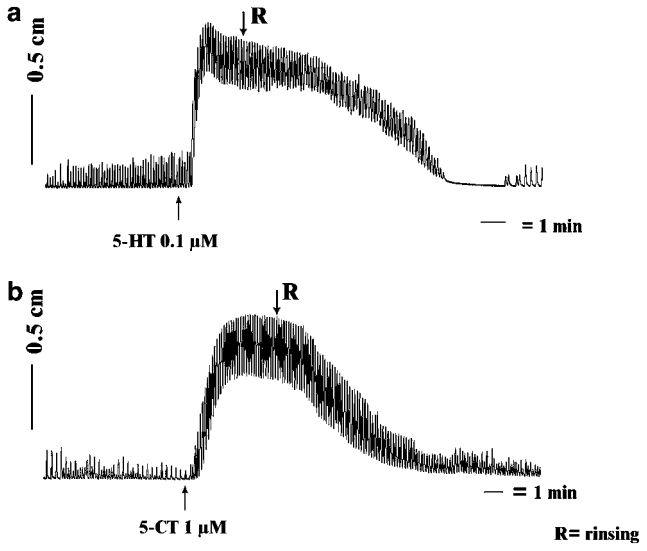

Figure 1 Representative tracings of the response to 0.1 μM 5-HT (a) and 1 μM 5-CT (b) in isolated equine jejunal longitudinal muscle strips.

it is necessary to constrain the agonist and antagonist concentration–effect curves to have the same maximum (in this case 0.1 μM). So, in the above-described logistic function $IC_{50'}$ is derived from the antagonist inhibition curves, which were constructed by nonlinear regression. The $EC_{50'}$ value was obtained by fitting the control concentration–response curve to 5-HT in 24 horses (Figure 1b) to a maximum of 0.1 μM 5-HT and constraining the Hill slope to 1.

All values are expressed as mean $\pm$ s.e.m.; n denotes the number of tissues obtained from different horses. Significance was set at a value of $P<0.05$.

Results

Concentration–response curves to 5-HT

The equine jejunal longitudinal muscle strips showed spontaneous phasic activity. 5-HT induced mainly a tonic contraction with superimposed phasic activity (Figure 1a). The amplitude and the frequency of these phasic contractions tended to be increased in comparison to the spontaneous activity before the administration of 5-HT, but this effect did not show concentration-dependency. Only the tonic response was therefore measured for calculation.

Figure 2a shows the concentration–response curves obtained by cumulative administration of 5-HT in the same strip and by administration of eight increasing concentrations of 5-HT to eight parallel strips. The cumulative concentration–response curve was bell shaped and the maximal effect was clearly decreased compared to that of the isolated curve. Accordingly, the cumulative administration protocol was not used to investigate the effect of 5-HT in equine jejunum longitudinal muscle.

Figure 2b shows the constructed mean isolated 5-HT (1 nM–10 μM) concentration–response curve of 24 horses. It has the features of a monophasic sigmoidal concentration–response curve, consistent with a single-site interaction. The iterative fitting procedure of the individual curves yields a mean upper

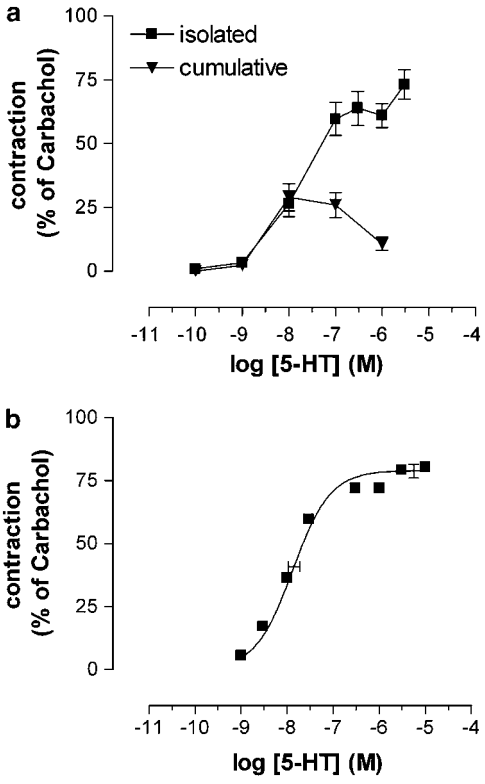

Figure 2 (a) Mean ($\pm$s.e.m) concentration–response curves to 5-HT, when added cumulatively or in an isolated way (eight increasing concentrations in eight different tissues) in equine jejunal longitudinal muscle ($n=6$). (b) Mean isolated concentration–response curve to 5-HT in equine jejunal longitudinal smooth muscle strips ($n=24$). The curve shown represents a simulation using the Hill equation; the estimates of E_{max} (with vertical error bars) and pEC_{50} (with horizontal error bars) are shown.

asymptote E_{max} of 79.04 $\pm$ 2.47%, a mean midpoint location pEC_{50} of 7.88 $\pm$ 0.07 and a mean Hill slope of 1.07 $\pm$ 0.08.

Effect of TTX, atropine and L-NNA on the response to 5-HT

Addition of TTX and atropine to the organ baths had no effect on frequency or amplitude of spontaneous activity or base-line tonus. The midpoint location, slope and upper asymptotes of the isolated concentration–response curves to 5-HT that served as a control were not significantly influenced by the combination of TTX (0.3 μM) and atropine (0.3 μM), (Table 1). The contractile response to 1 μM 5-HT in the presence of TTX (3 μM) or atropine (1 μM) or the combination of both was also not changed in comparison to the response induced by 1 μM 5-HT before adding TTX and/or atropine (Table 2).

L-NNA (100 μM), a nitric oxide synthase inhibitor, did not influence spontaneous activity or base-line tonus of the tissues. There was no significant effect on the concentration–response curve to 5-HT (Table 1).

Effect of 5-HT receptor antagonists on the response to 5-HT

Neither the selective 5-HT_{2A}-receptor antagonist ketanserin (0.3 μM; Hoyer *et al.*, 1994), nor the selective 5-HT_3 receptor antagonist granisetron (0.3 μM; Sanger & Nelson, 1989) altered

Table 1 Curve parameters for the isolated concentration–response curves to 5-HT and 5-CT in the absence and presence of the antagonists indicated

Agonist	*In the absence of antagonist*			*In the presence of antagonist*		
	E_{max} (%)	pEC_{50}	n_H	E_{max} (%)	pEC_{50}	n_H
5-HT						
TTX plus atropine ($n=4$) (both 0.3 μM)	90.44±2.31	7.56±0.23	0.88±0.14	89.29±2.88	7.66±0.11	0.83±0.19
L-NNA ($n=4$) (100 μM)	65.80±6.00	7.85±0.13	0.90±0.05	73.00±7.82	7.52±0.26	0.90±0.05
Ketanserin ($n=4$) (0.3 μM)	80.64±3.55	7.83±0.05	0.81±0.06	86.91±4.46	7.63±0.14	0.70±0.12
Granisetron ($n=4$) (0.3 μM)	85.40±2.57	8.26±0.08	1.30±0.15	82.26±0.84	8.15±0.11	1.04±0.13
GR 113808 ($n=4$) (0.1 μM)	75.46±4.12	7.92±0.08	1.01±0.16	76.98±4.16	7.87±0.10	0.84±0.07
SB 269970 ($n=4$) (0.3 μM)	70.94±7.60	7.72±0.11	0.91±0.15	72.22±8.18	7.52±0.07	0.74±0.07
5-CT						
TTX plus atropine ($n=4$) (both 0.3 μM)	86.90±7.48	6.19±0.26	0.74±0.06	86.16±7.71	6.34±0.31	0.73±0.09
SB 269970 ($n=4$) (0.3 μM)	86.90±7.48	6.19±0.26	0.74±0.06	83.99±3.19	6.31±0.27	0.69±0.06

Values are expressed as mean±s.e.m.

Table 2 Contractile responses to 1 μM 5-HT and 1 μM 5-CT before and in the presence of the antagonists indicated

Antagonist	*Response to 1* μM *5-HT*	
	Before	*In the presence*
TTX ($n=4$) (3 μM)	73.32±1.02	72.44±1.75
Atropine ($n=4$) (1 μM)	74.03±2.37	74.93±1.81
TTX plus atropine ($n=4$) (3 and 1 μM)	73.33±1.64	74.38±1.34
GR 127935 ($n=5$) (0.1 μM)	49.26±2.07	49.65±2.16
Ketanserin ($n=6$) (0.3 μM)	49.35±6.44	39.08±5.63
SB 204741 ($n=4$) (0.3 μM)	52.38±2.18	41.33±6.27
RS 102221 ($n=6$) (0.3 μM)	52.29±2.88	51.79±3.32
Granisetron ($n=6$) (0.3 μM)	57.72±3.16	52.94±4.17
GR 113808 ($n=6$) (0.1 μM)	58.08±3.91	56.99±5.15
	Response to 1 μM *5-CT*	
TTX ($n=4$) (3 μM)	68.42±3.71	68.54±2.71
Atropine ($n=4$) (1 μM)	66.98±0.35	69.61±1.58
TTX plus atropine ($n=4$) (3 and 1 μM)	65.91±2.68	65.97±2.16

Values are expressed as mean±s.e.m.

the concentration–response curve to 5-HT (Table 1). The same observation was made for the highly selective 5-HT_4 receptor antagonist GR 113808 (0.1 μM; Johnson *et al*., 1993) and the 5-HT_7 receptor antagonist SB 269970 (0.3 μM; Hagan *et al*., 2000).

In further experiments, antagonists were tested by studying the response to 1 μM 5-HT before and in the presence of a given antagonist within the same tissue. These experiments confirmed that the 5-HT_{2A} receptor antagonist ketanserin (0.3 μM), the 5-HT_3 receptor antagonist granisetron (0.3 μM) and the 5-HT_4 receptor antagonist GR 113808 (0.1 μM) had no significant influence on the response to 5-HT; they further showed that also the 5-$HT_{1B,D}$ receptor antagonist GR 127935 (0.1 μM; Terron, 1996), the 5-HT_{2B} receptor antagonist SB 204741 (0.3 μM; Forbes *et al*., 1995) and the 5-HT_{2C} receptor antagonist RS 102221 (0.3 μM; Bonhaus *et al*., 1997) did not significantly influence the response to 5-HT (Table 2).

The 5-HT_{1A} receptor antagonists NAN 190 (0.1, 0.3 and 1 μM; Cao & Rodgers, 1997) and WAY 100635 (3, 30 and 300 nM; Khawaja *et al*., 1995) inhibited the contractions to 5-HT in a concentration-dependent fashion. Figure 3 shows the results for NAN 190. Although the concentration–response curves of 5-HT in an individual horse showed a somewhat capricious shape, the mean results illustrate a parallel rightward shift of the concentration–response curve to 5-HT in the presence of increasing concentrations of NAN 190. The slopes and upper asymptotes of the concentration–response curves to 5-HT in the presence and the absence of NAN 190 were indeed not significantly different, while the pEC_{50} significantly decreased (Table 3). The pK_b values calculated on the basis of the results with 0.1, 0.3 and 1 μM NAN 190 were 7.58±0.51, 7.54±0.24 and 7.55±0.19, respectively. WAY 100635 (3 nM) shifted the concentration–response curve to 5-HT to the right in a parallel way without a change in E_{max}, but the higher concentrations of WAY 100635 (30 and 300 nM) significantly depressed the E_{max} of 5-HT (Figure 4, Table 3). Apparently, WAY 100635 behaves as a noncompetitive antagonist in these higher concentration ranges. The pK_b value calculated for the lowest concentration of WAY 100635 was 8.83±0.44.

In view of the capricious shape of the isolated concentration–response curves to 5-HT in the individual horses in Figures 3 and 4, and the fact that different antagonist concentrations were tested in different tissues in these experiments, the antagonists NAN 190 and WAY 100635 were also tested in different concentrations *versus* 0.1 μM 5-HT within the same tissue (Figures 5, 6). Both antagonists concentration-dependently reduced the response to 0.1 μM 5-HT; their effect was easily rinsed out. From these experiments, a pK_b value of 8.13±0.06 was estimated for NAN 190 and a pK_b value of 8.69±0.07 for WAY 100635, using the 'functional' version of the Cheng–Prusoff equation proposed by Lazareno & Birdsall (1993).

The 5-HT_1, 5-HT_2, 5-HT_5, 5-HT_6 and 5-HT_7 receptor antagonist methysergide (1, 10 and 100 nM; Gommeren *et al*., 1998) antagonised nonsurmountably the 5-HT-induced concentration–response curve as shown in Figure 7. When tested in different concentrations (0.2–3.2 nM) *versus* 0.1 μM 5-HT in the same tissues, methysergide induced a concentration-dependent reduction of the response to 5-HT; this effect was only partially washed out (Figure 6).

Influence of other 5-HT receptor agonists

In accordance with the 5-HT response, it was shown in preliminary experiments that the cumulative concentration–response curve to the 5-HT_1, 5-HT_7 receptor agonist 5-CT (Hoyer *et al*., 1994) was clearly depressed at the higher

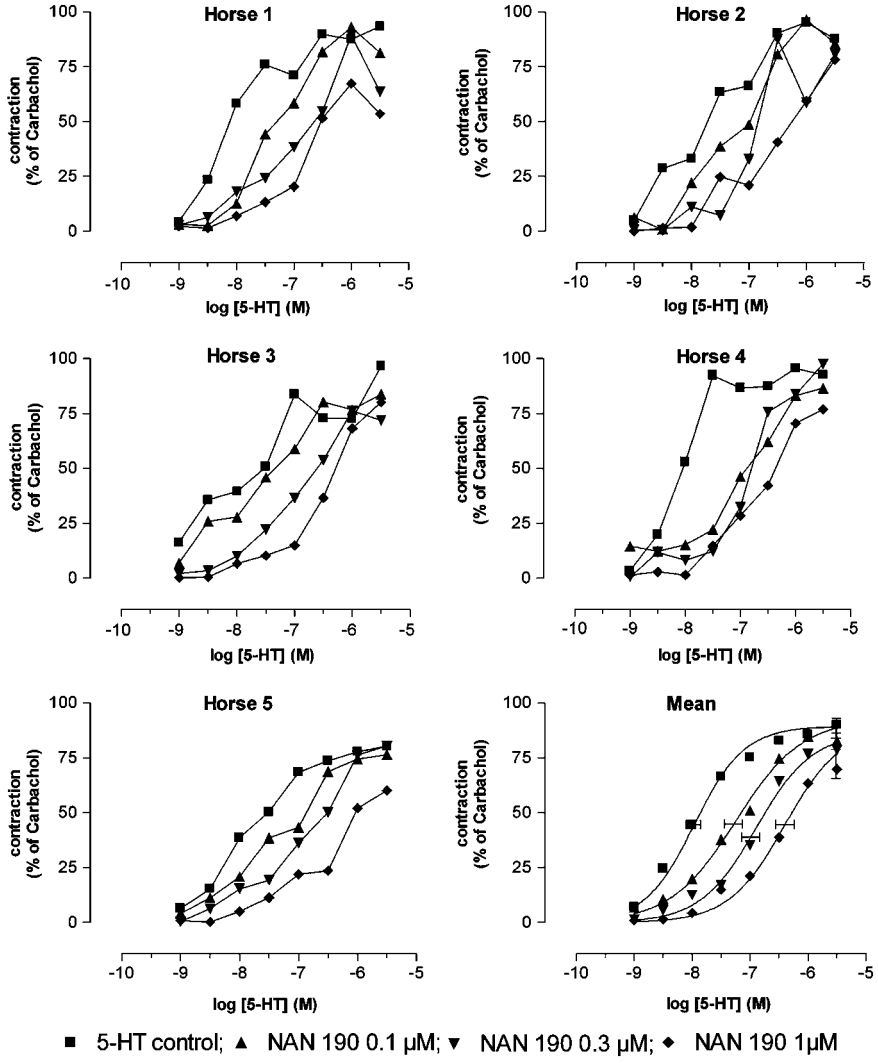

Figure 3 Influence of increasing concentrations of NAN 190 on the 5-HT-induced contraction of equine jejunal longitudinal muscle strips. The individual responses in five different horses (horse 1–5) are shown, as well as the mean curve simulations using the Hill equation; in the latter panel, the estimates for E_{max} (with vertical error bars) and pEC_{50} (with horizontal error bars) are given.

Table 3 Curve parameters for the isolated concentration–response curves to 5-HT and 5-CT in the absence and presence of increasing concentrations of NAN 190 (5-HT) or WAY 100635 (5-HT, 5-CT)

	E_{max}	pEC_{50}	n_H
5-HT control	89.32±3.00	7.96±0.09	1.02±0.23
NAN 190 (0.1 μM)	92.74±4.74	7.24±0.16*	0.76±0.11
NAN 190 (0.3 μM)	86.41±5.39	6.89±0.08**	0.90±0.11
NAN 190 (1 μM)	88.92±9.18	6.40±0.17**	0.90±0.16
5-HT control	83.55±3.04	7.53±0.17	0.83±0.10
WAY 100635 (3 nM)	80.96±6.28	7.13±0.08*	0.99±0.21
WAY 100635 (30 nM)	73.00±3.53*	6.60±0.10**	0.72±0.12
WAY 100635 (300 nM)	56.10±10.47*	6.35±0.21**	1.22±0.16*
5-CT control	85.86±3.78	6.20±0.13	0.63±0.09
WAY 100635 (3 nM)	84.22±1.90	5.81±0.09*	0.76±0.11
WAY 100635 (30 nM)	66.22±5.44*	5.78±0.14**	1.10±0.07*
WAY 100635 (300 nM)	33.35±6.85**	5.48±0.21**	1.30±0.13*

Values are expressed as mean±s.e.m ($n=5$–6).
*$P<0.05$, **$P<0.001$: significantly different *versus* 5-HT or 5-CT in the absence of antagonist.

concentrations of 5-CT in comparison to the isolated one, so that only isolated concentration–response curves were obtained in further experiments with 5-CT.

Addition of 5-CT to the organ baths elicited a response similar to that of 5-HT (Figure 1b). The contractile responses to 5-CT are concentration-dependent, yielding curve

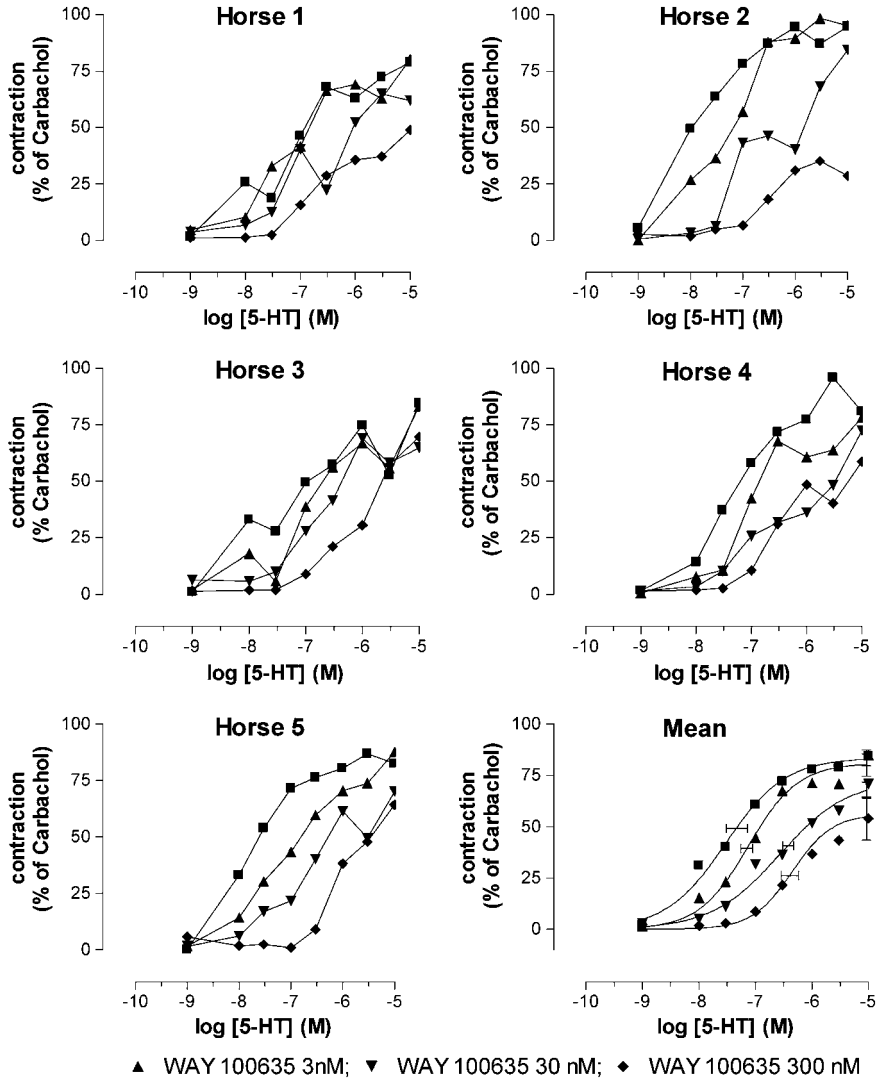

Figure 4 Influence of increasing concentrations of WAY 100635 on the 5-HT-induced contraction of equine jejunal longitudinal muscle strips. The individual responses in five different horses (horse 1–5) are shown, as well as the mean curve simulations using the Hill equation; in the latter panel, the estimates for E_{max} (with vertical error bars) and pEC_{50} (with horizontal error bars) are given.

parameters for E_{max} of 85.86±3.78%, pEC_{50} of 6.02±0.18 and a mean Hill slope of 0.76±0.09.

In contrast, the 5-HT_{1A} receptor agonists 8-OH-DPAT (Sanger & Schoemaker, 1992) and flesinoxan (Hadrava *et al*., 1995), and the partial 5-HT_{1A} receptor agonist buspiron (Sharif *et al*., 2004) had no effect in the muscle strips (tested concentrations: 1 nM to 3 μM). When 5-HT (1 μM) was added on top of 8-OH-DPAT, flesinoxan or buspiron, this immediately induced muscle strip contraction. 8-OH-DPAT, flesinoxan and buspiron did not antagonise the response to 5-HT. The contractile response to 0.1 μM 5-HT was 66.16±6.20% before and 70.99±8.69% in the presence of 0.1 μM 8-OH-DPAT, 64.43±5.56% before and 68.70±5.14% in the presence of 0.1 μM flesinoxan, and 68.38±4.88% before and 63.15±7.47% in the presence of 1 μM buspiron ($n=6$ for each series); the response to 5-HT in the parallel control tissues not receiving antagonist was 72.23±3.24 and 79.57±4.68% ($n=6$).

Effect of antagonists on the response to 5-CT

Curve parameters of the concentration–response curves to 5-CT were not influenced by application of TTX plus atropine (both 0.3 μM), nor by the selective 5-HT_7 receptor antagonist SB 269970 (0.3 μM) (Table 1). Likewise, the contractile response to 1 μM 5-CT in the presence of TTX (3 μM) or atropine (1 μM) or the combination of both was also not changed in comparison to the response induced by 1 μM 5-CT before adding TTX and/or atropine (Table 2).

The specific 5-HT_{1A} receptor antagonist WAY 100635 in its lowest concentration (3 nM) produced a parallel rightward shift of the concentration–contraction curve to 5-CT, without influence on the maximum response (Figure 8; Table 3). When WAY 100635 was applied at a concentration of 30 nM and 0.3 μM, the concentration–response curve to 5-CT was further shifted to the right but there was a clear concomitant suppression of the maximum effect elicited by 5-CT. The pK_b value calculated for the lowest concentration of WAY 100635 was 8.63±0.34.

Discussion

Interaction of 5-HT with muscular 5-HT receptors, antagonised by the 5-HT$_{1A}$ receptor antagonists NAN 190 and WAY 100635

The inability of TTX and atropine, even in the higher concentrations tested, to affect the 5-HT-induced contractile response in equine jejunal longitudinal smooth muscle suggests that 5-HT mediates its effects through non-neurogenic, nonholinergic pathways. A similar mechanism of action was observed in the circular smooth muscle of the equine jejunum (Nieto *et al*., 2000). Also a possible interference of NO release by 5-HT was excluded by the lack of effect of the NO synthase inhibitor L-NNA on the 5-HT-induced contractile response.

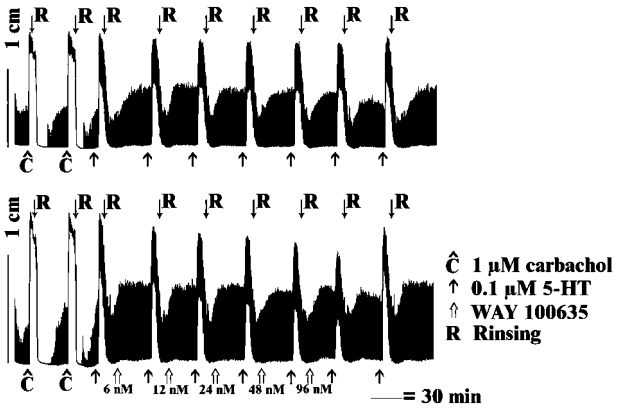

Figure 5 Representative traces showing the influence of 1 μM carbachol and 0.1 μM 5-HT in two equine jejunum longitudinal muscle strips. In the upper panel, 5-HT was studied seven times consecutively without adding an antagonist (control); in the lower panel, the response to 5-HT was studied in the presence of increasing concentrations of WAY 100635.

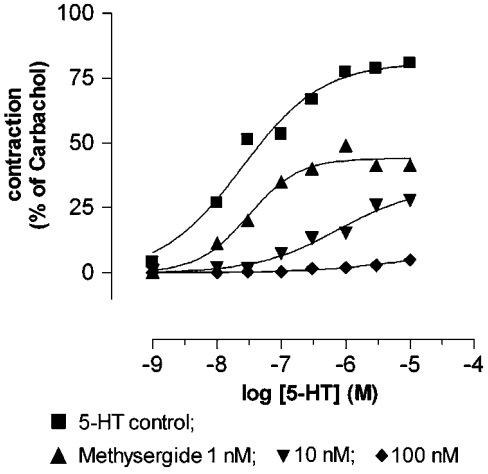

Figure 7 Concentration–response curves to 5-HT in the absence and the presence of increasing concentrations of methysergide in equine jejunal longitudinal muscle strips ($n=5$). The curves shown represent simulations using the Hill equation.

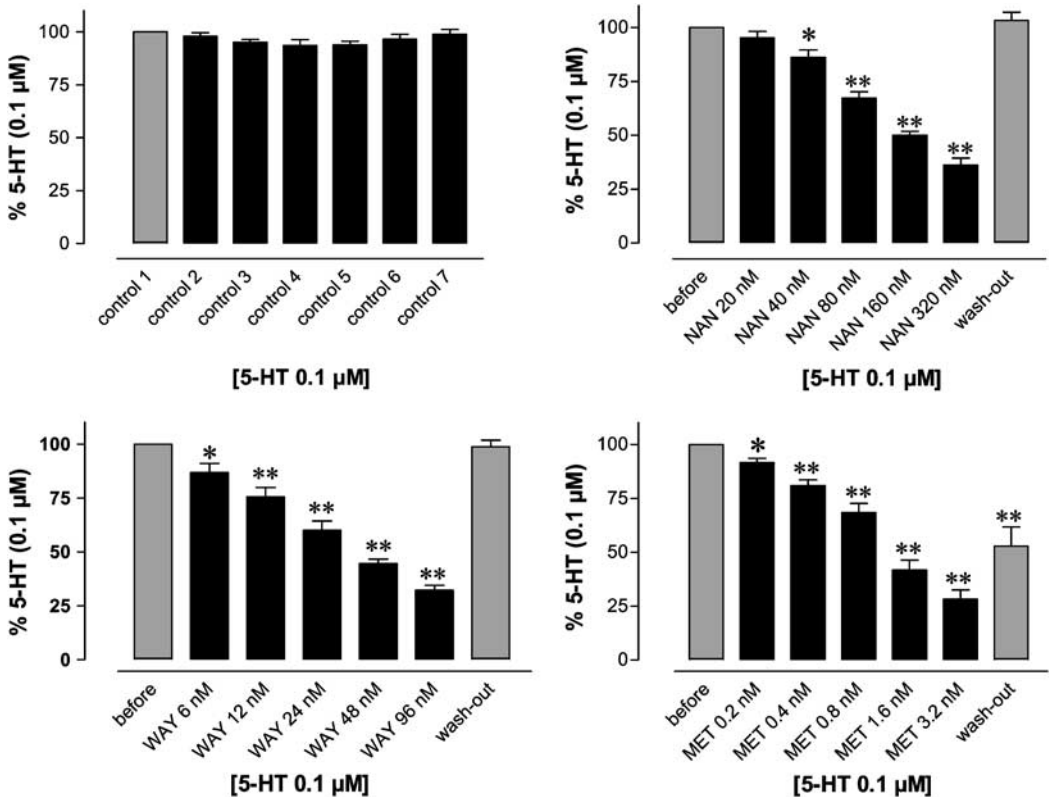

Figure 6 Influence of increasing concentrations of NAN 190 (NAN), WAY 100635 (WAY) and methysergide (MET) on the 5-HT-induced (0.1 μM) response of equine jejunal longitudinal muscle strips. 5-HT was administered seven times at 30 min interval; antagonists were added 20 min before the second to sixth administration. Tissues were rinsed after each 5-HT-induced contraction; the seventh administration was performed to check for wash out of the antagonists. In control strips, 5-HT was tested seven times without adding antagonist (left upper panel). Data are expressed as mean values $\pm$ s.e.m ($n=8$). *$P<0.05$; **$P<0.001$ *versus* 5-HT before antagonist.

From the experiments in which several antagonists were tested *versus* a full 5-HT concentration–response curve, or *versus* a single nearly maximal concentration of 5-HT, the participation of 5-$HT_{1B,1D}$, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_4 and 5-HT_7 receptors in the 5-HT-induced contractile response can be excluded. These findings on longitudinal muscle are in contrast with those on 5-HT-induced responses in equine jejunal circular smooth muscle, where interaction with 5-HT_2 and 5-HT_3 receptors has been proposed (Nieto *et al*., 2000).

Of all tested antagonists, only the 5-HT_{1A} receptor antagonists NAN 190 and WAY 100635, and the 5-$HT_{1,2,5,6,7}$ receptor antagonist methysergide elicited a clearcut inhibitory effect on the 5-HT-induced contractile response of equine jejunum longitudinal smooth muscle. The specific 5-HT_{1A} receptor antagonist NAN 190 fulfiled all requirements of pure competitive antagonism. The pK_b calculated from the experiments, where increasing concentrations of NAN 190 were tested *versus* a fixed concentration of 5-HT (8.13 ± 0.06), is in good accordance with the affinity of NAN 190 for the 5-HT_{1A} receptor, reported in the literature (Ahlers *et al*., 1992: pigeon brain, p$K_b = 8.12$; Sharif *et al*., 2004: human cloned 5-HT_{1A} receptors, p$K_b = 8.5$). The pK_b calculated from the experiments with concentration–response curves of 5-HT was more than a half unit lower (7.54–7.58). We have no explanation for this difference.

The second 5-HT_{1A} receptor antagonist WAY 100635 (3, 30 and 300 nM) also concentration-dependently antagonised the

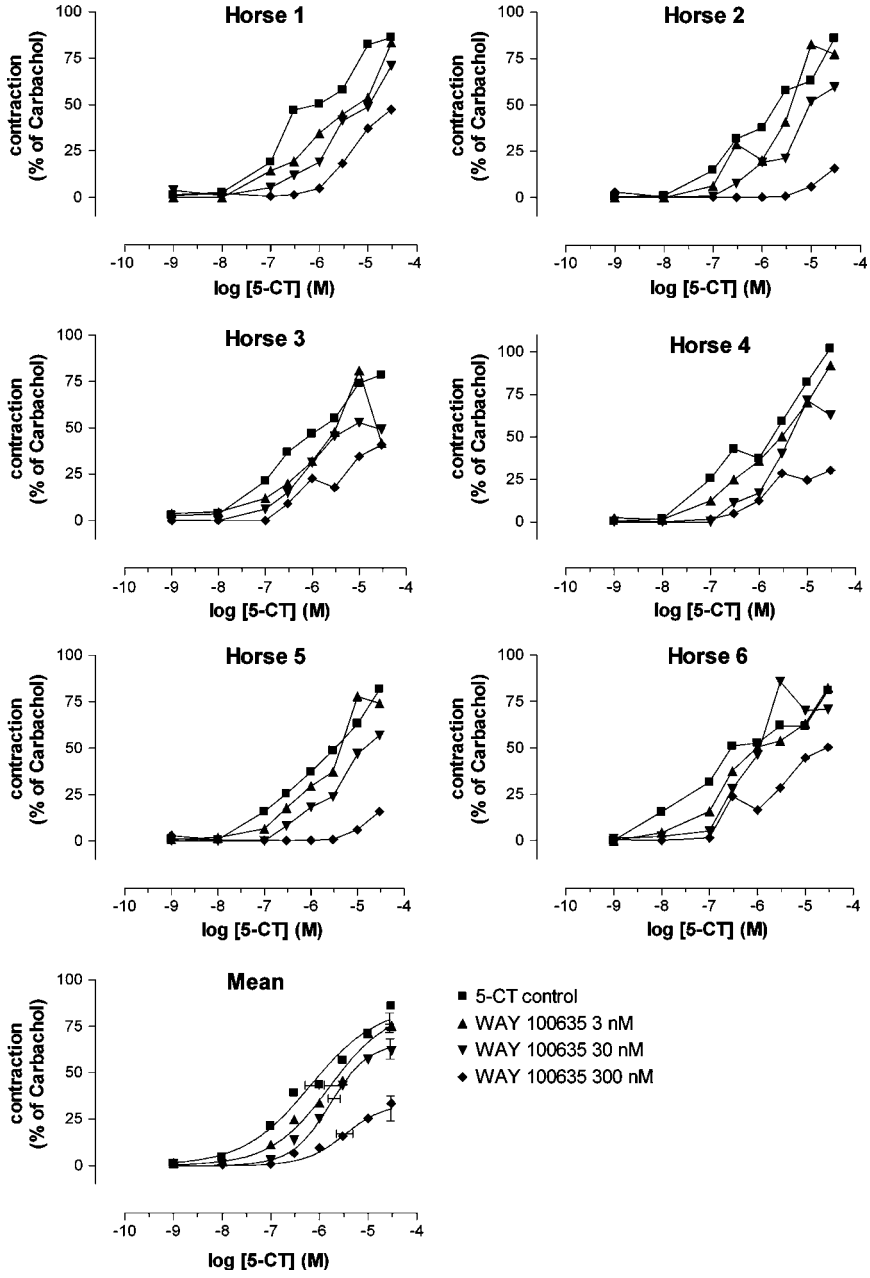

Figure 8 Influence of increasing concentrations of WAY 100635 on the 5-CT-induced contraction of equine jejunal longitudinal muscle strips. The individual responses in six different horses (horse 1–6) are shown, as well as the mean curve simulations using the Hill equation; in the latter panel, the estimates for E_{max} (with vertical error bars) and pEC_{50} (with horizontal error bars) are given.

contractile responses to 5-HT but from 30 nM on, it behaved as a noncompetitive antagonist, decreasing the maximal effect of 5-HT. A pK_b estimate was calculated from the experiments where five concentrations of WAY 100635 were tested *versus* 0.1 μM 5-HT. It should be realised that the pK_b calculated in this way can be to some extent an overestimation of the antagonising effect of WAY 100635 as the decrease in the 5-HT-induced response by WAY 100635 is not solely determined by competitive antagonism. Still the pK_b estimate obtained (8.69 ± 0.07) was similar to that calculated for the lowest concentration of WAY 100635 *versus* the concentration–response curve of 5-HT (8.83 ± 0.44); these values correspond to pK_b values reported before for WAY 100635 at 5-HT_{1A} receptors (Fletcher *et al*., 1994: rat hippocampal 5-HT_{1A} receptors, $pIC_{50} = 8.87 \pm 0.14$; Khawaja *et al*., 1997: CHO cell line transfected with human recombinant 5-HT_{1A} receptors, $pIC_{50} = 8.39 \pm 0.12$; Hall *et al*., 1997: human brain, p$K_b = 8.60$). All these cited *in vitro* studies were performed in brain tissue, the principal location of 5-HT_{1A} receptors. In these tissues, WAY 100635 behaves as a pure competitive antagonist. However, in one study on a gastrointestinal myenterically localised 5-HT_{1A} receptor, WAY 100635 behaved as a competitive antagonist of 5-CT when tested in electrically stimulated guinea-pig ileum up to a concentration of 0.3 nM, but showed insurmountable antagonism at higher concentrations (Forster *et al*., 1995). The results with NAN 190 and WAY 100635 thus seem to point to an interaction of 5-HT with 5-HT_{1A} receptors in equine jejunal longitudinal smooth muscle. This seems corroborated by the results with the 5-$HT_{1,7}$ receptor agonist 5-CT.

As for the 5-HT-induced contractile response, it was observed that TTX and atropine did not influence the effect of 5-CT. Owing to the lack of effect of the 5-HT_7 receptor antagonist SB 269970, the 5-CT-induced motor effects point to activation of 5-HT_1 receptors, located directly on the smooth muscle cells. The influence of WAY 100635 on the concentration–response curve of 5-CT was similar to its effect on 5-HT and the pK_b calculated for the lowest concentration of WAY 100635, which influenced the concentration–response curve of 5-CT in a competitive way, was similar to that obtained for 5-HT (8.63 *versus* 8.83), supporting the interaction of 5-CT and 5-HT with the same receptor.

The presence of a gastrointestinal muscular 5-HT_{1A} receptor would be exceptional. The 5-HT_{1A} receptor is found predominantly in the central nervous system, the hippocampus and neocortex (Pazos & Palacios, 1985; Moller *et al*., 2004). 5-HT_{1A} receptors are only occasionally described in the gastrointestinal tract and when a gastrointestinal localisation was identified, they reside in neuronal tissue where they mediate inhibitory functions. In the myenteric plexus of the isolated guinea-pig ileum and stomach, the neuronally localised 5-HT_{1A} receptors mediate inhibition of electrically evoked twitch contractions (Bill *et al*., 1990; Buchheit & Buhl, 1994; Lepard & Galligan, 2004). *In situ* hybridisation reveals that many submucosal and myenteric neurons of the rat and guinea-pig small intestine express mRNA encoding the 5-HT_{1A} receptor (Kirchgessner *et al*., 1993; 1996). The response of enteric neurons to 5-HT that has been attributed to 5-HT_{1A} receptors is a hyperpolarisation, accompanied by an increase in input resistance caused by an increase in K^+ conductance (Galligan *et al*., 1988). Inhibitory enteric 5-HT_{1A} receptors have also been located on nerve terminals releasing the mediators of fast and slow excitatory postsynaptic potentials. Inhibition of synaptic transmission in the myenteric plexus is likely to account for 5-HT-induced inhibition of the peristaltic reflex in some studies (Galligan, 1996). Indeed, 5-HT_{1A} receptor activation has been found to induce inhibition of acetylcholine release from the guinea-pig myenteric plexus (Dietrich & Kilbinger, 1996). In contrast, a gastrointestinal muscular 5-HT_{1A} receptor is expected to induce an excitatory contractile response, when coupled to inhibition of adenylate cyclase. This is indeed the primary coupling mechanism of this receptor, although also other coupling mechanisms are described (Raymond *et al*., 1999).

Differences between the receptor mediating the contractile effect of 5-HT in equine jejunum and the 5-HT_{1A} receptor

Although the results with NAN 190 and WAY 100635 *versus* 5-HT and 5-CT suggest the presence of a 5-HT_{1A} receptor in equine jejunum, several observations do not fit with this conclusion.

(1) 5-CT is expected to be equipotent with 5-HT or even more potent than 5-HT at 5-HT_{1A} receptors (Newman-Tancredi *et al*., 1998; Cowen *et al*., 2005). However, in equine jejunum longitudinal muscle, 5-CT was at least 10-fold less potent than 5-HT.
(2) Methysergide has been shown to possess agonist activity (Pauwels *et al*., 1993; Hoyer *et al*., 1994) and to have a low affinity (Kilpatrick *et al*., 1989) at 5-HT_{1A} receptors. However, in equine jejunum, methysergide had no contractile effect *per se* and seemed to have a high affinity at the receptor involved, having a pronounced antagonising effect at 1 nM. It can be mentioned that methysergide was shown to antagonise the inhibitory effect of 5-HT *via* 5-HT_{1A} receptors on electrically induced GABA release from GABAergic neurones in the guinea-pig ileum, but in a concentration of 300 nM (Shirakawa *et al*., 1989).
(3) Three specific 5-HT_{1A} receptor agonists, that is, 8-OH-DPAT, buspiron and flesinoxan, did not elicit any contractile effect in the equine jejunum. They also did not antagonise the effect of 5-HT. In a system with low efficacy reserve, a partial 5-HT_{1A} receptor agonist such as buspiron (Pauwels *et al*., 1993; Sharif *et al*., 2004) might stay without effect *per se*, but it should antagonise the effect of the full agonist 5-HT, which was not the case.

It is thus clear that the receptor involved in the contractile effect of 5-HT and 5-CT in equine jejunal longitudinal muscle does not correspond with a classic 5-HT_{1A} receptor. This might be related to the presence of another 5-HT receptor subtype, not yet described. Alternatively, a possible explanation could be found in interspecies differences in the specific structure of the 5-HT_{1A} receptor. As a member of the 5-HT_1 family of serotonin receptors, the 5-HT_{1A} receptor is a seven-transmembrane spanning receptor, composed of 422 amino acids. The rat and human 5-HT_{1A} receptor nucleic acid sequences are 88% homologous with each other and accordingly there appears to be a similar pharmacological profile observed between these species (Raymond *et al*., 1999). The 5-HT_{1A} receptor has one antagonist-binding site and five different agonist-binding sites (Raymond *et al*., 1999). Restricted mutations can lead to very important changes in the effect of a given substance. When Guan *et al*.

(1992) mutated Asn^{386} in the seventh transmembrane domain of the human 5-HT_{1A} receptor, this caused a 100-fold decline in the affinity of the antagonist pindolol binding to the 5-HT_{1A} receptor. Ho *et al*. (1992) rendered the 5-HT_{1A} receptor refractory to 5-HT stimulation in several ways by introducing various point mutations. The substitution of a conserved asparagine at position 396 (localised in the seventh transmembrane region) with either alanine, phenylalanine or valine results in a 5-HT_{1A} receptor that is refractory to 8-OH-DPAT activation (Chanda *et al*., 1993).

It can be concluded that the muscular contractile 5-HT receptor in equine jejunal longitudinal muscle cannot be characterised between the actually known classes of 5-HT receptors with the experimental data provided, but is sensitive to the 5-HT_{1A} receptor antagonists NAN 190 and WAY 100635.

Desensitization of the equine muscular 5-HT receptor

In the former studies concerning *in vitro* characterization of 5-HT-induced responses in the equine gut, it is not mentioned whether it was tested that the applied cumulative administration protocol of 5-HT yielded the same contractile responses as isolated administration (Nieto *et al*., 2000; Weiss *et al*., 2002). In our study, apparently a fast desensitisation of the muscular 5-HT receptors takes place. It can be mentioned that desensitisation is a typical feature of the 5-HT_{1A} receptor (Raymond *et al*., 1999; Serres *et al*., 2000; Hensler & Durgam, 2001). Acute treatment with 5-HT_{1A} agonists leads to rapid desensitisation of central 5-HT_{1A} autoreceptors (Beer *et al*., 1990; Seth *et al*., 1997; Riad *et al*., 2001). Rapid desensitisation of 5-HT_{1A} receptors by agonists has also been described in various transfected cell lines (Nebigil *et al*., 1995; Rotondo *et al*., 1997; Della Rocca *et al*., 1999). Whether we are dealing with an 'equine' 5-HT_{1A} receptor or another not yet characterised 5-HT receptor, our observation of a rapidly desensitising muscular 5-HT receptor in the equine jejunum opens interesting considerations concerning the possible role of this receptor in the complex pathophysiology of ileus in colic horses, where several factors can serve as a possible source of 5-HT overload. Bailey *et al*. (2003) already identified the presence of bioactive amines formed by bacterial decarboxylation of amino acids in the caecum and colon of healthy and colic horses. It is known that the permeability of intestinal mucosa in horses is increased during intestinal ischaemia, which promotes translocation of endotoxins and possibly dietary amines, among which 5-HT, from the chyme into the systemic circulation (Snyder, 1989; Morris, 1991; Bailey *et al*., 2000; 2004; Vatistas *et al*., 2003). Within the scope of research into the ethiopathogenesis of laminitis in horses, it was shown that during i.v. administration of *Escherichia coli* lipopolysacharids for experimental induction of endotoxemia, a clear increase in plasma 5-HT and thromboxane beta 2 levels is seen. Both substances are released during activation of blood platelets (Elliott *et al*., 2003; Vatistas *et al*., 2003; Menzies-Gow *et al*., 2004). Therefore, important amounts of 5-HT can be released into the blood stream in colic horses with ischaemic or necrotic intestinal segments. These increased 5-HT levels in ileus horses might lead to desensitisation of the muscular 5-HT receptor, meaning that 5-HT can no longer stimulate the smooth muscle cells *via* these receptors. In how far the muscular contractile 5-HT receptor might contribute to hypomotility in ileus has to be further investigated.

Conclusion

This study shows the presence of muscular 5-HT receptors, inducing contraction in equine jejunal longitudinal muscle. The receptor does not belong to the 5-$HT_{1B,1D}$, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_4 and 5-HT_7 receptor class. Although blocked by the 5-HT_{1A} receptor antagonists NAN 190 and WAY 100635, the receptor cannot be classified as a classic 5-HT_{1A} receptor since the 5-HT_{1A} receptor agonists 8-OH-DPAT, flesinoxan and buspiron were not active. Whether a horse-specific 5-HT_{1A} receptor or a not yet described 5-HT receptor subtype is involved needs further investigation. More research is also needed to clarify whether these muscular contractile 5-HT receptors play a role in the pathophysiology of ileus and/or can serve as pharmacological target for possible prokinetic medication in horses.

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