GASTROINTESTINAL MOTILITY, PROKINETIC BENZAMIDES AND SEROTONIN

A STUDY ON THE GUINEA-PIG COLON

Wageningen

18 MEI 1995

UB-CARDEX

CENTRALE LANDBOUWCATALOGUS

0000 0577 2070

904791

Promotor:	dr L.M.A. Akkermans hoogleraar Neurofysiologie der Vertebraten (Fysiologie van Mens en Dier, LUW)
Co-promotor:	dr J.A.J. Schuurkes hoofd Afdeling Gastro-Intestinale Farmacologie (Janssen Research Foundation, Beerse, België)

NN08201, 1940

MICHEL RAYMOND BRIEJER

GASTROINTESTINAL MOTILITY, PROKINETIC BENZAMIDES AND SEROTONIN

A STUDY ON THE GUINEA-PIG COLON

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE LANDBOUW- EN MILIEUWETENSCHAPPEN
OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR C.M. KARSSEN
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 2 JUNI 1995
DES NAMIDDAGS TE VIER UUR IN DE AULA
VAN DE LANDBOUWUNIVERSITEIT TE WAGENINGEN

bsn=904791

Promotiecommissie

promotor:	Prof. dr L.M.A. Akkermans, Fysiologie van Mens en Dier, Landbouwuniversiteit Wageningen
co-promotor:	Dr J.A.J. Schuurkes, Afdeling Gastro-intestinale Farmacologie, Janssen Research Foundation
overige leden:	Prof. dr P.R. Saxena, Vakgroep Farmacologie, Erasmus Universiteit Rotterdam
	Prof. dr F.P. Nijkamp, Vakgroep Farmacologie, Universiteit Utrecht
	Prof. dr J.H. Koeman, Vakgroep Toxicologie, Landbouwuniversiteit Wageningen
referent:	Prof. dr R.A. Lefebvre, Universiteit Gent

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Briejer, Michel Raymond

GASTROINTESTINAL MOTILITY, PROKINETIC BENZAMIDES AND SEROTONIN : A STUDY ON THE GUINEA-PIG COLON / Michel Raymond Briejer. - [S.l. : s.n.] - Ill. Thesis Landbouw Universiteit Wageningen. With index, ref. - With summary in Dutch.

ISBN 90-5485-388-3

Subject headings: pharmacology / serotonin / gastroenterology

Printed by ICG printing bv - Dordrecht. The printing of this thesis was financially supported by the Janssen Research Foundation.

BIBLIOTHEEK
LANDBOUWUNIVERSITEIT
Wageningen

NN08201, 1940

Stellingen

1. De rol van niet-adrenerge, niet-cholinerge neurotransmitters in het maag-darmkanaal bij het werkingsmechanisme van prokinetische benzamiden zou wel eens onderschat kunnen zijn. *dit proefschrift*

2. Stimulatie van 5-HT_4-receptoren in de plexus myentericus verklaart slechts ten dele de prokinetische werking van de gesubstitueerde benzamiden.
dit proefschrift

3. Afwezigheid van een remming van een respons door tetrodotoxine duidt niet *per se* op een direct effect op de gladde-spiercellen, daar een deel van de door neuronen gemedieerde effecten in het maag-darmkanaal ongevoelig is voor tetrodotoxine.
Hirst GDS & Spence I (1973) Nature 243: 54-56; North RA (1973) Br J Pharmacol 49: 709-710; dit proefschrift

4. De hypothese, dat niet alleen facilitatie van vrijzetting van contraherende, maar ook van relaxerende neurotransmitters belangrijk is voor de werking van prokinetische benzamiden, verdient nader onderzoek. *dit proefschrift*

5. De stelling dat 5-HT_4-receptoren uitsluitend op de enterische zenuwuiteinden en niet op het cellichaam voorkomen, is onvoldoende gefundeerd en niet noodzakelijkerwijs waar. *Tonini M et al. (1991) Pharmacol Res 24: 5-14; dit proefschrift*

6. De wetenschappelijke ogen worden bijna volledig gericht op NO, maar de twintig jaar eerder ontdekte neurotransmitter ATP krijgt terecht thans ook weer aandacht. *dit proefschrift*

7. De studies, waarop de hypothese is gebaseerd dat dopamine een neurotransmitter is in het maag-darmkanaal, vormen in het licht van de huidige kennis een onvoldoede gefundeerde basis voor deze hypothese.
Willems JL et al., (1985) Pharmacol Rev 37: 165-216

8. De karakterisering van neurotransmitter-receptoren dient bij voorkeur te gebeuren met behulp van agonisten en antagonisten met een structuur die niet wezenlijk afwijkt van die van de endogene agonist.
Leff P & Martin GR (1988) Med Res Rev 8: 187-202

9. Dat de rol van de longitudinale spierlaag van het maag-darmkanaal in maag-darmmotiliteit meestal onderbelicht wordt duidt op een gebrek aan kennis over de functie van deze laag.

10. De relatief geringe belangstelling die het wetenschappelijk onderzoek naar de motoriek van het maag-darmkanaal krijgt, is waarschijnlijk het gevolg van enerzijds de schijnbare eenvoud van bouw en functie, en anderzijds de onaantrekkelijke aard van haar inhoud.

dr A.J.P.M. Smout, oratie ter gelegenheid van de aanvaarding van het hoogleraarschap Pathofysiologie en Kliniek van de Gastro-intestinale Motoriek aan de UU, 9-11-1994

11. Voor farmacologische contractie-relaxatie-studies in orgaanbaden met geïsoleerde weefsels zouden bij voorkeur condities en weefsels moeten worden gekozen opdat zowel relaxatie als contractie gemeten kan worden, teneinde maskeringseffecten te kunnen opmerken.

12. De beschouwing dat eten niets anders is dan je over je voedsel heen schuiven getuigt van een grove onderschatting van de complexiteit van de gastro-intestinale motiliteit.

13. Politisering van de wetenschap is een rem op haar vooruitgang.

14. Hoe ouder een verondersteld feit in de farmacologische wetenschappen is, des te groter is de kans dat zij onwaar of achterhaald is.

15. Gelet op de geringe selectiviteit van nagenoeg elke 5-HT-receptorligand is het onverstandig om uit *in-vivo*-studies, op grond van met een beperkt aantal van dergelijke stoffen verkregen informatie, stellige conclusies te trekken met betrekking tot het type 5-HT-receptor, dat is betrokken bij het bestudeerde fenomeen.

Stellingen behorend bij het proefschrift **Gastrointestinal motility, prokinetic benzamides and serotonin**. A study on the guinea-pig colon. Michel R. Briejer Wageningen, 2 juni 1995.

To Nicole
To my parents

Abstract

Briejer, M.R. (1995) Gastrointestinal motility, prokinetic benzamides and serotonin. A study on the guinea-pig colon. PhD Thesis, Department of Human and Animal Physiology, Wageningen Agricultural University, Wageningen, The Netherlands.

The prokinetic substituted benzamides stimulate gastrointestinal motility in animal models and in man. Studies done on the isolated guinea-pig ileum have led to the hypothesis that the benzamides act through facilitation of cholinergic transmission due to stimulation of serotonin$_4$ (5-HT$_4$) receptors on the intramural enteric neurons. However, many questions as to their mode of action remain. The substituted benzamides are known to interfere with a variety of 5-HT receptor types. In this thesis the pharmacological action and interaction of substituted benzamides and 5-HT on the guinea-pig proximal colon were studied. This preparation was found to be endowed with 5-HT$_3$ and 5-HT$_4$ receptors on the nerves, mediating contraction *via* the release of acetylcholine and a non-cholinergic neurotransmitter (probably substance P, which stimulates smooth muscle NK$_1$ receptors), and 5-HT$_{2A}$ receptors on the smooth muscle. The benzamides cisapride and R 76 186 were found to be agonists at this 5-HT$_4$ receptor, but cisapride was also found to have an additional direct effect on the smooth muscle. Furthermore, an unknown neuronal 5-HT$_2$-like receptor was found to mediate relaxation involving nitric oxide (NO) and adenosine triphosphate (ATP). Cisapride and some other benzamides were found to selectively enhance the ATP-mediated relaxation. It is thus proposed that the benzamides do not only facilitate the excitatory cholinergic transmission, but also the inhibitory purinergic transmission. As in peristalsis coordinated contraction and relaxation are important, the benzamides might thus promote peristalsis by enhancing both relaxation and contraction on demand. An *in vivo* model for faecal pellet propulsion in the guinea-pig distal colon was developed to investigate this hypothesis in future studies.

Table of contents

General introduction 9

Chapter 1 On the mechanism of action of gastrointestinal prokinetic substituted benzamides. 19

Chapter 2 Pharmacological characterization of 5-hydroxytryptamine receptor types in the guinea-pig proximal colon. 65

Chapter 3 Substance P-induced contractions of the guinea-pig proximal colon through stimulation of post-junctional tachykinin NK_1 receptors. 81

Chapter 4 Cisapride and a structural analogue, R 76 186, are 5-hydroxytryptamine$_4$ (5-HT_4) receptor agonists on the guinea-pig colon ascendens. 89

Chapter 5 Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*. 105

Chapter 6 5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide. 121

Chapter 7 Novel 5-HT_2-like receptor mediates neurogenic relaxation on the guinea-pig proximal colon. 143

Chapter 8 Cisapride and structural analogs selectively enhance 5-hydroxytryptamine (5-HT) -induced purinergic transmission in the guinea pig proximal colon. 163

Chapter 9 A novel *in vivo* method for the assessment of peristalsis in the distal colon of the guinea-pig. 181

General discussion

Summary

Samenvatting

List of publications

Dankwoord/Acknowledgements

Curriculum vitae

General introduction

Serotonin (5-hydroxytryptamine; 5-HT) is abundantly present in the gut. It is contained in high concentrations in the enterochromaffin cells (Erspamer, 1966; Racké & Schwörer, 1991), and its presence in neurons of the enteric nervous system has been demonstrated (Costa *et al.*, 1982; Furness & Costa, 1982; Legay *et al.*, 1984; see also Gershon *et al.*, 1989). 5-HT plays a role as a neurotransmitter (see: Gershon & Erde, 1981; Gershon *et al.*, 1990) in secretion processes (Cussato *et al.*, 1982; Johnson *et al.*, 1994) and gastrointestinal motility (see: Gershon *et al.*, 1990; Dhasmana *et al.*, 1993). Furthermore, 5-HT is involved in the regulation of blood flow (see: Parks & Jacobson, 1987) influences vascular permeability, and it is contained in mast cells, suggesting a role in the immune system of the gut (see: Kraneveld, 1994).

The effects to 5-HT are mediated by a wide array of 5-HT receptor types. It has been shown, that in the guinea-pig[1] gastrointestinal tract, 5-HT_{1A}, 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors are involved in motor responses to 5-HT (see: Gershon *et al.*, 1990; Dhasmana *et al.*, 1993). 5-HT_{1A}, 5-HT_3 and 5-HT_4 receptors are located on the enteric nerves and regulate the release of excitatory neurotransmitters, e.g. acetylcholine and the neurokinin substance P (Fox & Morton, 1991; Kilbinger & Wolf, 1992; Broad *et al.*, 1993; Pan *et al.*, 1994; Ramirez *et al.*, 1994) (see Figure I). Other 5-HT receptors have been shown to be present on the smooth muscle of the guinea-pig gut: 5-HT_{2A} receptors mediate contraction of the ileum (Engel *et al.*, 1984), and stimulation of "orphan" 5-HT receptors causes relaxation in the stomach fundus and ileum[2] (Feniuk *et al.*, 1983; Kalkman *et al.*, 1986; Kojima *et al.* 1992) (see Figure I). Furthermore, putative 5-HT_{1P} receptors[3] and other orphan 5-HT receptors have been shown to exist in the gut of this species. In the guinea-pig colon, Kojima (1991) and Elswood & Bunce (1992) demonstrated the presence of an unknown neuronally located 5-HT receptor, which mediates the release of a relaxing substance. Currently, several neurotransmitters are considered to be a candidate of non-adrenergic non-cholinergic (NANC) inhibitory nerves, e.g. adenosine triphosphate (ATP) (Burnstock, 1972), vaso-

---

[1] Historically, most studies on neuronal projections, neurotransmitter contents of neurons and electrophysiological, functional and pharmacological studies have been done in the guinea-pig. Especially the ileum has been subject of research.

[2] By "orphan" receptors it is indicated that these receptors cannot be classified according to the current classification criteria. The pharmacological profile of the orphan receptor in the guinea-pig ileum mediating myogenic relaxation resembles that of the recently cloned 5-ht$_7$ receptor, whose mRNA has been shown to be present in the gut.

[3] 5-HT_{1P} receptor-mediated effects have only been found in electrophysiological experiments and are as yet poorly defined from a pharmacological point of view; as such, their naming is not (yet) recognized by the Receptor Nomenclature Committee of the Serotonin Club.

active intestinal polypeptide (VIP) (Goyal *et al.*, 1980) and nitric oxide (NO) (Bult *et al.*, 1990). It has however not been investigated which of these substances are involved in the 5-HT-induced relaxation in the guinea-pig colon.

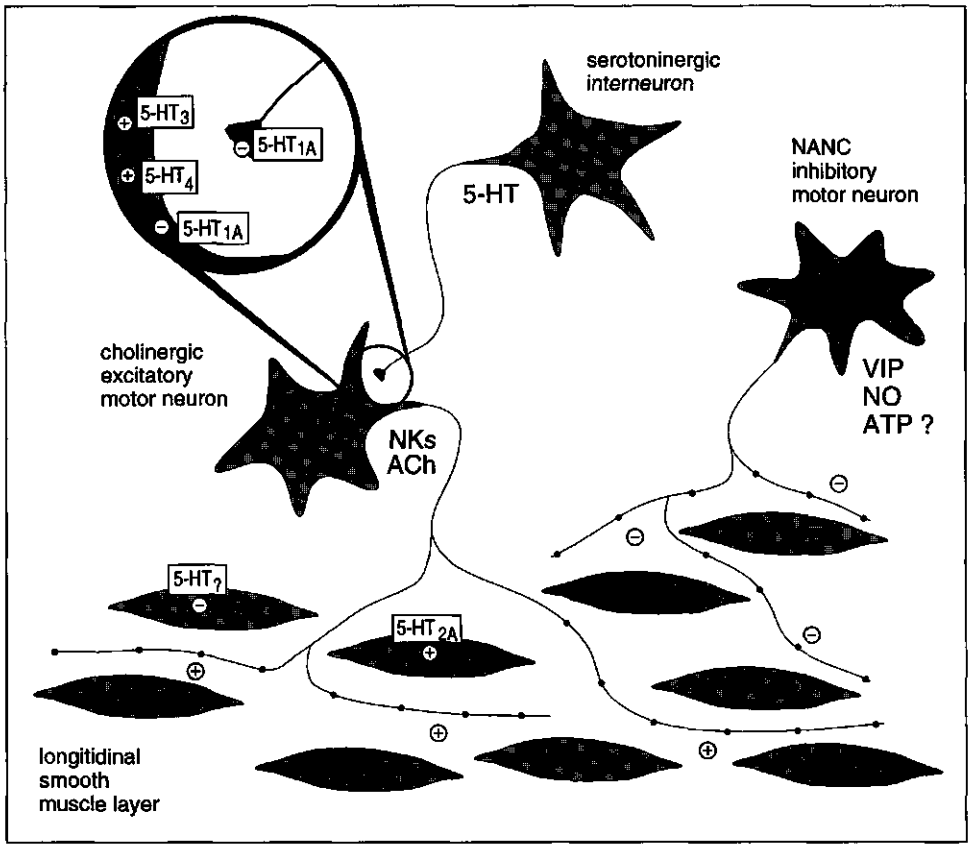


Figure 1 Schematic representation of the proposed innervation of the guinea-pig ileum. The longitudinal muscle layer and some myenteric neurons are depicted. The scheme has been greatly simplified as only the excitatory serotoninergic, cholinergic and inhibitory non-adrenergic non-cholinergic (NANC) neurons are shown. Serotonin (5-HT) released from interneurons and other consecutively released neurotransmitters can stimulate a variety of both neuronal and smooth muscle receptors in the ileum causing either stimulation (+) or inhibition (-). After stimulation of 5-HT_3 and 5-HT_4 receptors, the release of acetylcholine (ACh) and neurokinins (NKs) is enhanced. The NANC inhibitory neurotransmitters are likely to include vasoactive intestinal polypeptide (VIP), nitric oxide (NO) and possibly adenosine triphosphate (ATP) or related purines. On the smooth muscle, 5-HT_{2A} and unknown 5-HT receptors are present.

Drugs with a substituted benzamide moiety[4] (see Table 1.1) are used in the treatment of impaired gastrointestinal motility, which manifests in disorders like gastro-esophageal reflux, gastroparesis, functional dyspepsia and constipation (Reyntjens *et al.*, 1986; Müller-Lissner, 1987; Reynolds & Putnam, 1992). For some decades, metoclopramide (see: Pinder *et al.*, 1976) has been used, but is has now been largely superseded by cisapride (Wiseman & Faulds, 1994), while clebopride and cinitapride[5] are also applied in some countries (Massingham *et al.*, 1985; Reynolds & Putnam, 1992). Other prokinetic[6] benzamides are represented by dazopride, renzapride and zacopride, which are not therapeutically used (Reynolds & Putnam, 1992; Gullikson *et al.*, 1992).

The mechanism of action of the prokinetic benzamides is to date still poorly understood. Already in 1970, Bianchi *et al.* (1970) observed that metoclopramide inhibited contractions to 5-HT on the guinea-pig ileum, an effect which was later shown to be mediated by 5-HT_3 receptors. Two decades later, a 5-HT receptor type was characterized in mouse colliculi neurons in primary culture (Dumuis *et al.*, 1988), and consecutively also in guinea-pig ileum and colon, rat oesophagus and heart atrium of several species (see: Bockaert *et al.*, 1992; Ford & Clarke, 1993). It was found that stimulation of these 5-HT_4 receptors, as they were soon called, in guinea-pig gut tissues causes facilitation of cholinergic (and possibly also non-cholinergic) excitatory transmission (Schuurkes *et al.*, 1985; Elswood *et al.*, 1991; Bingham & Andrews, 1992; Kilbinger & Wolf, 1992). The prokinetic benzamides were found to be relatively specific (partial) agonists at these 5-HT_4 receptors (Dumuis *et al.*, 1989; Bockaert *et al.*, 1990; Taniyama *et al.*, 1991).

The 5-HT_4 receptor agonist action of the prokinetic benzamides has been put forward as the mechanism by which motility is enhanced (see: Tonini *et al.*, 1991), and the non-benzamide 5-HT_4 receptor agonist BIMU 1 has an action *in vivo* which is similar to that of benzamides (Rizzi *et al.*, 1994). However, several papers have appeared, suggesting that other mechanisms could be important as well. De Ridder & Schuurkes reported that in the dog gastric antrum *in vitro*, cisapride enhanced field stimulation-induced cholinergic contractions *via* a mechanism that was not sensitive to 5-HT_4 receptor blockade (De Ridder & Schuurkes, 1993). In the human colon, cisapride did even not enhance the

---

[4] 2-Methoxy-4-chloro-5-amino-substitution for most prokinetic benzamides; these compounds differ significantly from the antipsychotic benzamides, which are potent dopamine D_2 receptor antagonists, while possessing no prokinetic activity.

[5] 2-Ethoxy-4-nitro-5-amino substitution on the benzene ring.

[6] A prokinetic drug is a substance that enhances co-ordinated gastrointestinal motility and promotes propulsion.

release of acetylcholine (Burleigh & Trout, 1985). In this tissue, 5-HT_4 receptors are located on the smooth muscle and mediate inhibition. Nevertheless, cisapride stimulates colonic motility and can help to treat mild constipation (Müller-Lissner, 1987; Reynolds & Putnam, 1992). With metoclopramide, sensitization to acetylcholine has been observed in the isolated guinea-pig colon (Beani *et al.*, 1970), and similar results have later been obtained by other workers with different benzamides and tissues. In electrophysiological experiments with guinea-pig myenteric neurons, direct 5-HT_4 receptor-mediated effects could not be demonstrated, though cisapride has been shown to enhance cholinergic excitatory postsynaptic potentials, suggesting an increased release of acetylcholine (Nemeth *et al.*, 1985; Mawe *et al.*, 1986; Tonini *et al.*, 1989; Wade *et al.*, 1991, Pan & Galligan, 1994). In similar experiments, 5-HT can induce a slow prolonged depolarization *via* the stimulation of putative 5-HT_{1P} receptors. Some of the prokinetic benzamides interfere with this 5-HT_{1P} receptor-mediated response, either as agonist (S-zacopride) or antagonist (cisapride, R-zacopride, renzapride) (Nemeth *et al.*, 1985; Mawe *et al.*, 1986; 1989; Wade *et al.*, 1991). Enhancement of gastric emptying in mice has been associated with antagonism of 5-HT_{1P} receptors on inhibitory neurons (Mawe *et al.*, 1989) but other reports on functional effects mediated by 5-HT_{1P} receptors are not available. Hence, it is clear that many mechanisms additional to 5-HT_4 recepor agonism could account for the prokinetic action of benzamides.

In many investigations, the actions of 5-HT and benzamides have been studied on the isolated guinea-pig ileum, using it either as an intact segment or as a preparation of the longitudinal smooth muscle layer together with the myenteric nerves. The colon however shows pharmacological and physiological characteristics that are very different from those of the ileum. For example, in contrast to the longitudinal muscle of the ileum, the colon actively develops a resting tone, allowing the study of both relaxation and contraction. After electrical transmural stimulation, the larger part of the relaxation-contraction response of the longitudinal muscle is mediated by NANC transmitters, while in the ileum the contraction is mainly cholinergic in nature (Bianchi *et al.*, 1968; Furness, 1970; Galligan, 1993). Also at receptor level, there are striking differences. For example, the relaxation-mediating orphan 5-HT receptor (see footnote 2) on the ileum is located on the smooth muscle, while on the colon orphan receptors are located on the enteric nerves. Thus, the colon might reveal novel pharmacological characteristics of the substituted benzamides.

The **aims** of the investigations described in this thesis, were to establish:

- *in the isolated guinea-pig colon:*
  1. which 5-HT receptor types mediate the responses to 5-HT, and what their location is (on the smooth muscle or on the enteric nerves);
  2. which neurotransmitters are involved in the relaxing (NO, ATP, VIP) and contracting (acetylcholine, substance P) actions of 5-HT after neuronal 5-HT receptor stimulation;
  3. the action of substance P and determine the receptor type that mediates this action;
  4. the contraction to cisapride and a structural analog, R 76 186[7], and to investigate how their effects are mediated;
  5. the exact 5-HT receptor subtype *via* which 5-HT induces neurogenic relaxations;
  6. whether and how cisapride interferes with the 5-HT-induced relaxations;

- *in the guinea-pig colon in vivo:*
  7. a new *in vivo* model for the study of peristalsis in the distal colon.

[7] Contractions to R 76186 are fully blocked by tetrodotoxin while those to cisapride are inhibited merely partially. Thus, R 76 186 could provide a tool for better understanding the effects of cisapride.

References

Beani L, Bianchi C, Crema C (1970) Effects of metoclopramide on isolated guinea-pig colon. 1) Peripheral sensitization to acetylcholine. *Eur J Pharmacol* 12: 320-331.

Bianchi C, Beani L, Frigo GM, Crema A (1968) Further evidence for the presense of non-adrenergic inhibitory structures in the guinea-pig colon. *Eur J Pharmacol* 4: 51-61.

Bianchi C, Beani L, Crema A (1970) Effects of metoclopramide on isolated guinea-pig colon. 2) Interference with ganglionic stimulant drugs. *Eur J Pharmacol* 12: 332-341.

Bingham S, Andrews PLR (1992) Gastrointestinal motility effects of cisapride mediated by non-adrenergic, noncholinergic nerves. In: *Gastrointestinal dysmotility: focus on cisapride* (Heading RC, Wood JC, Eds.), New York, Raven Press Ltd.: pp.93-106.

Bockaert J, Sebben M, Dumuis A (1990) Pharmacological characterization of 5-hydroxytryptamine$_4$ (5-HT$_4$) receptors positively coupled to adenylate cyclase in adult guinea pig hippocampal membranes: effects of substituted benzamide derivatives. *J Pharmacol Exp Ther* 37: 408-411.

Bockaert J, Fozard JR, Dumuis A, Clarke DE (1992) The 5-HT$_4$ receptor: a place in the sun. *Trends Pharmacol Sci* 13: 141-145.

Broad RM, McDonald TJ, Cook MA (1993) Adenosine and 5-HT inhibit substance P release from nerve endings in myenteric ganglia by distinct mechanisms. *Am J Physiol* 264: G454-G461.

Bult H, Boeckxstaens PA, Pelckmans PA, Jordaens FH, Van Maercke YM, Herman AG (1990) Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* 345: 346-347.

Burleigh DE, Trout SJ (1985) Evidence against an acetylcholine releasing action of cisapride in the human colon. *Br J Clin Pharmacol* 20: 475-478.

Burnstock G (1972) Purinergic nerves. *Pharmacol Rev* 24: 509-581.

Costa M, Furness JB, Cuello AC, Verhofstad AAJ, Steinbusch HDJ, Elde RP (1982) Neurons with 5-hydroxytryptamine-like immunoreactivity in the enteric nervous system: their vizualization and reactions to drug treatment. *Neuroscience* 7: 351-363.

Cussato J, Jodal M, Tuttle R, Lundgren O (1982) 5-Hydroxytryptamine and cholera secretion. *Scand J Gastroenterol* 17: 695-703.

De Ridder WE, Schuurkes JAJ (1993) Cisapride and 5-hydroxytryptamine enhance motility in the canine antrum *via* separate pathways, not involving 5-hydroxytryptamine$_{1,2,3,4}$ receptors. *J Pharmacol Exp Ther* 264: 79-88.

Dhasmana KM, Zhu YN, Cruz SL, Villalón CM (1993) Gastrointestinal effects of 5-hydroxytryptamine and related drugs. *Life Sci* 53: 1651-1661.

Dumuis A, Bouhelal R, Sebben M, Cory R, Bockaert J (1988) A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol Pharmacol* 34: 880-887.

Dumuis A, Sebben M, Bockaert J (1989) The gastrointestinal prokinetic benzamide derivatives are agonists at the non-classical 5-HT receptor (5-HT$_4$) positively coupled to adenylate cyclase in neurons. *Naunyn-Schmiedeberg's Arch Pharmacol* 340: 403-410.

Elswood CJ, Bunce KT, Humphrey PPA (1991) Identification of putative 5-HT$_4$ receptors in guinea-pig ascending colon. *Eur J Pharmacol* 196: 149-155.

Elswood CJ, Bunce KT (1992) Investigation of the 5-HT receptor mediating relaxation in guinea-pig ascending colon. *J Pharm Pharmacol* 44: 264-266.

Engel G, Hoyer D, Kalkman HO (1984) Identification of 5-HT$_2$ receptors on longitudinal muscle of the guinea-pig ileum. *J Receptor Res* 4: 113-126.

Erspamer V (1966) Occurence of indolealkyl amines in nature. In: *Handbuch der experimentellen Pharmakologie* (Echler O, Fara A Eds.), Springer Verlag, Berlin: pp.132-181

Feniuk W, Humphrey PPA, Watts AD (1983) 5-Hydroxytryptamine-induced relaxation of mammalian smooth muscle. *Eur J Pharmacol* 96: 71-78.

Ford APDW, Clarke DE (1993) The 5-HT_4 receptor. *Med Res Rev* 13: 633-662.

Fox AJ, Morton IKM (1991) An examination of the proposed functional interaction of substance P and 5-HT in the guinea pig ileum. *Ann New York Acad Sci* 632: 385-387.

Furness JB (1970) An examination of the nerve-mediated, hyoscine-resistant excitation of the guinea-pig colon. *J Physiol* 207: 803-821.

Furness JB, Costa M (1982) Neurons with 5-hydroxytryptamine-like immunoreactivity in the enteric nervous system: their projections in the guinea-pig small intestine. *Neuroscience* 7: 341-349.

Galligan JJ (1993) Differential inhibition of cholinergic and noncholinergic neurogenic contractions by mu opioid and alpha-2 adrenergic agonists in guinea pig ileum. *J Pharmacol Exp Ther* 264: 365-383.

Gershon MD, Erde SM (1981) The nervous sustem of the gut. *Gastroenterology* 80: 1571-1594.

Gershon MD, Mawe GM, Branchek TA (1989) 5-Hydroxytryptamine and enteric neurons. In: *The peripheral actions of 5-hydroxytryptamine* (Fozard JR Ed.), Oxford-New York-Tokyo: Oxford University Press: pp.247-273.

Gershon MD, Wage PR, Kirchgessner AL, Tamir H (1990) 5-HT Receptor subtypes outside the central nervous system. Roles in the physiology of the gut. *Neuropsychopharmacology* 3: 385-395.

Goyal RK, Rattan S, Said S (1980) VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory nerves. *Nature* 288: 378-380.

Gullikson GW, Virina MA, Loeffler RF, Yang DC, Goldstin B, Flynn DL, Moormann AE (1992) Gastrointestinal motility responses to the S and R enantiomers of zacopride, a 5-HT_4 agonist and 5-HT_3 antagonist. *Drug Dev Res* 26: 405-417.

Johnson PJ, Bornstein JC, Furness JB, Woollard DJ, Orrman-Rossiter SL (1994) Characterization of 5-hydroxytryptamine receptors mediating mucosal secretion in guinea-pig ileum. *Br J Pharmacol* 111: 1240-1244.

Kalkman HO, Engel G, Hoyer D (1986) Inhibition of 5-carboxamidotryptamine-induced relaxation of guinea-pig ileum correlates with [125I]LSD binding. *Eur J Pharmacol* 129: 139-145.

Kilbinger H, Wolf D (1992) Effects of 5-HT_4 receptor stimulation on the basal and electrically evoked release of acetylcholine from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch Pharmacol* 345: 270-275.

Kojima S (1991) Characterization of 5-hydroxytryptamine-induced relaxations of guinea-pig proximal colon. *Arch Int Pharmacodyn* 313: 23-32.

Kojima S, Ishizaki R, Shimo Y (1992) Investigation into the 5-hydroxytryptamine-induced relaxation of the circular smooth muscle of the guinea-pig stomach fundus. *Eur J Pharmacol* 224: 45-49.

Kraneveld AD (1994) Neuroimmune regulation of microvascular premeability. Central role of the mast cell. PhD Thesis, Faculty of Pharmacy, Utrecht University, The Netherlands.

Legay C, Saffrey MJ, Burnstock G (1984) Coexistence of immunoreactive substance P and serotonin in neurons of the gut. *Brain Res* 302: 379-382.

Massingham R, Bou J, Roberts DJ (1985) A comparison of the stimulatory effects of metoclopramide and cinitapride in the guinea-pig isolated ileum. *J Auton Pharmacol* 5: 41-53.

Mawe GM, Branchek TA, Gershon MD (1986) Peripheral neural serotonin receptors: identification and characterization with specific antagonists and agonists. *Proc Natl Acad Sci USA* 83: 9799-9803.

Mawe GM, Branchek TA, Gershon MD (1989) Blockade of 5-HT-mediated enteric slow EPSPs by BRL 24969: gastrokinetic effects. *Am J Physiol* 257: G386-G396.

Müller-Lissner SA (1987) Treatment of chronic constpation with cisapride and placebo. *Gut* 28: 1033-1038.

Nemeth PR, Ort, CA, Zafirov DH, Wood JD (1985) Interactions between serotonin and cisapride on myenteric neurons. *Eur J Pharmacol* 108: 77-83.

Pan H. Galligan JJ (1994) 5-HT_{1A} and 5-HT_4 receptors mediate inhibition and facilitation of fast synaptic transmission in enteric neurons. *Am J Physiol* 266: G230-G238.

Parks DA, Jacobson ED (1987) Mesenteric circulation. In: *Physiology of the gastrointestinal tract, second edition* (Johnson LR, Ed.) Raven Press, New York.

Pinder RM, Brogden RN, Sawyer PR, Speight TM, Avery GS (1976) Metoclopramide: a review of its pharmacological properties and clinical use. *Drugs* 12: 81-131.

Racké K, Schwörer H (1991) Regulation of serotonin release from the intestinal mucosa. *Pharmacol Res* 23: 13-25.

Ramirez MJ, Cenarruzabeitia E, Del Rio J, Lasheras B (1994) Involvement of neurokinins in the non-cholinergic response to activation of 5-HT_3 and 5-HT_4 receptors in guinea pig ileum. *Br J Pharmacol* 111: 419-424.

Reynolds JC, Putnam PE (1992) Prokinetic agents. *Gastroenterol Clin North Am* 21: 567-596.

Reyntjens A, Verlinden M, Aerts T (1986) Development and clinical use of the new gastrointestinal prokinetic drug cisapride (R 51619). *Drug Dev Res* 8: 251-265.

Rizzi CA, Sagrada A, Schiavone A, Schiantarelli P, Cesana R, Schiavi GB, Ladinsky H, Donetti A (1994) Gastroprokinetic properties of the benzimidazolone derivative BIMU 1, as agonist at 5-hydroxytryptamine$_4$ and antagonist at 5-hydroxytryptamine$_4$ receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* 349: 338-345.

Schuurkes JAJ, Van Nueten JM, Van Daele PGH, Reyntjens AJ, Janssen PAJ (1985) Motor stimulating properties of cisapride on isolated gastrointestinal preparations of the guinea-pig. *J Pharmacol Exp Ther* 234: 775-783.

Taniyama K, Nakayama S, Takeda K, Matsuyama S, Shirakawa J, Sano I, Tanaka C (1991) Cisapride stimulates motility of the intestine *via* the 5-hydroxytryptamine receptors. *J Pharmacol Exp Ther* 258: 1098-1104.

Tonini M, Galligan JJ, North RA (1989) Effects of cisapride on cholinergic neurotransmitters and propulsive motility in the guinea pig ileum. *Gastroenterology* 96: 1257-1264.

Tonini M, Rizzi CA, Manzo L, Onori L (1991) Novel enteric 5-HT_4 receptors and gastro-intestinal prokinetic action. *Pharmacol Res* 24: 5-14.

Wade PR, Mawe GM, Branchek TA, Gershon MD (1991) Use of stereoisomers of zacopride to analyze actions of 5-hydroxytryptamine on enteric neurons. *Am J Physiol* 260: G80-G90.

Wiseman LR, Faulds D (1994) Cisapride. An updated review of its pharmacology and therapeutic efficacy as a prokinetic agent in gastrointestinal motility disorders. *Drugs* 47: 116-152.

Chapter 1

On the Mechanism of Action of Gastrointestinal Prokinetic Substituted Benzamides

M.R. Briejer, L.M.A. Akkermans and J.A.J. Schuurkes

(in preparation)

Introduction

Substituted benzamides (Table 1.1) are currently widely used to treat motility disorders like gastro-esophageal reflux (heartburn), gastroparesis, functional dyspepsia and constipation (Reyntjens *et al.*, 1986; Müller-Lissner, 1987; Reynolds & Putnam, 1992). The very first benzamide to be used for this indication was metoclopramide. Nowadays, the most widely applied prokinetic benzamide (a prokinetic drug is a compound that enhances gastrointestinal propulsion and co-ordinated motility) is cisapride, though cinitapride and clebopride are also available in some countries (Table 1.1). Currently, there are some novel benzamides being evaluated in clinical trials, e.g. mosapride and lintopride (Table 1.1). Though the benzamide derivatives are known now for three decades to be effective gastrointestinal motility stimulants, still many questions as to their mechanism of action remain. In this review it is attempted to give an overview of the pharmacological data that have been obtained from *in vitro* and *in vivo* experiments as well as from studies in man. As different theories on the mechanism of action of prokinetic benzamides have been proposed during the last decades, a large part of this review places data in a historical perspective.

The development of metoclopramide

The local anaesthetic procaine (4-aminobenzoic acid 2-(diethylamino) ethyl ester) has served as the lead compound for the development of metoclopramide. Chemical modification of the procaine structure, making it less susceptible to hydrolysis, yielded procainamide (ester moiety changed into amide moiety), which showed increased anti-arrhythmic and local anaesthetic activity, and which also had some anti-emetic effects. These anti-emetic effects were found to be more pronounced if the benzene ring was chlorinated. Further substitution of the benzene ring by Delagrange chemists produced metoclopramide (which is an acronym for 2-methoxy-5-chloro-procainamide) (Table 1.1). Metoclopramide was found to have negligible local anaesthetic and anti-arrhythmic activity (Justin-Besançon *et al.*, 1964). Instead, the compound antagonized apomorphine-induced emesis and it was serendipitously found to improve upper gut motility (Justin-Besançon & Laville, 1964). As this improvement of motility (Jacoby & Brodie, 1967) was recognised to differ in a positive sense from drugs with similar actions known in those days (e.g. bethanechol, a cholinoceptor agonist), the therapeutic value of metoclopramide was soon appreciated. Today still, metoclopramide is

used to treat gastrointestinal symptoms related to impaired motility, and to tackle emesis (see for review on metoclopramide: Pinder *et al.*, 1976; Schulze-Delrieu, 1979).

The anti-emetic properties of metoclopramide have been explained to be caused by its anti-dopaminergic properties, i.e. by blocking dopamine D_2 receptors in the area postrema (see: Pinder *et al.*, 1976). However, not all types of emesis are dopamine-mediated, as McRitchie *et al.* (1984) pointed out, and they attempted to explain how metoclopramide antagonizes cisplatin and radiation-induced emesis in man. It has been suggested that its weak serotonin 5-HT_3 receptor antagonistic properties are responsible for the blocking of cisplatin-induced emesis in the ferret and man (see: Sanger & King, 1988).

Many mechanisms have been put forward in the 1970s and 1980s to explain the prokinetic action of metoclopramide. In 1975 a structural analogue to metoclopramide, clebopride, was synthesized. Clebopride is a more potent dopamine D_2 receptor antagonist, as is the chemically unrelated domperidone synthesized one year before, in 1974 (Laduron & Leysen, 1979; Roberts, 1982). As both compounds have a prokinetic action similar to metoclopramide (Brogden *et al.*, 1982; Roberts, 1982; Schuurkes *et al.*, 1982), is was soon hypothesized that the antidopaminergic properties were responsible for these effects.

The dopaminergic theory

Metoclopramide crosses the blood-brain barrier only to a minor extent, while clebopride does so easily (Niemegeers *et al.*, 1980). Domperidone, however, has been shown not to cross the blood-brain barrier (Laduron & Leysen, 1979; Niemegeers *et al.*, 1980). If it is supposed that metoclopramide, clebopride and domperidone stimulate gastrointestinal motility *via* a common mechanism, i.e. the blocking of dopamine D_2 receptors, these receptors must thus be localized in areas unprotected by the blood-brain barrier, e.g. on peripheral nerves.

The motility of the stomach and intestine is controlled autonomously by the intrinsic enteric nervous system, which is modulated by extrinsic sympathetic and parasympathetic nerves. In such extrinsic nerves, catecholamine histofluorescence and dopamine beta-hydroxylase immunoreactivity have been detected suggesting that these nerves contain noradrenergic and possibly also dopaminergic neurons (Gershon & Erde, 1981). The enteric nerves are however completely devoid of catecholamines (except in the guinea-pig colon) (Gershon & Erde, 1981).

Table 1.1 Structural formulas of the currently known gastro-intestinal motility stimulating substituted benzamides with their respective manufacturer and developmental status.

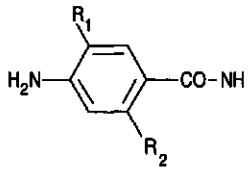
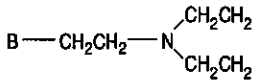
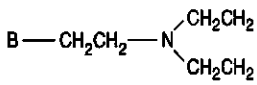
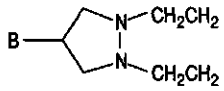
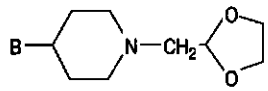
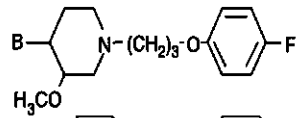
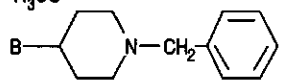
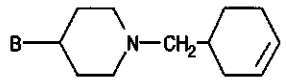
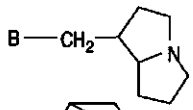
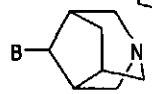
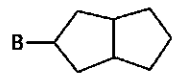
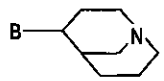
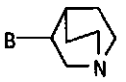
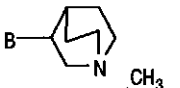
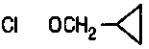
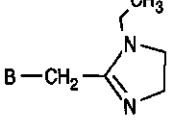
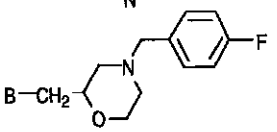
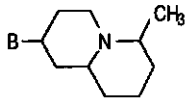
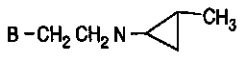
		R_1	R_2	
B:				
metoclopramide (Delagrange)		Cl	OCH_3	marketed in the early 1970s
bromopride (Synthelabo)		Br	OCH_3	launched (1976)
dazopride (Robins)		Cl	OCH_3	discontinued (1990)
dubopride (Fordonal/Almirall)		Cl	$O(CH_2)_3CH_3$	discontinued (1992)
cisapride (Janssen)		Cl	OCH_3	launched (1988)
clebopride (Almirall)		Cl	OCH_3	launched early 1980s
cinitapride (Almirall)		NO_2	OCH_2CH_3	launched (1990)
SC-53116/55822 /49518 (Searle)		Cl	OCH_3	preclinical
SC-50410/52491 (Searle)		Cl	OCH_3	preclinical (1992)
SC-52246 (Monsanto)		Cl	OCH_2CH_3	preclinical (1992)
renzapride (SmithKline Beecham)		Cl	OCH_3	available for licensing (1990)

Table 1.1 *Continued*

zacopride (Robins)		Cl	OCH_3	discontinued (1992)
pancopride (Almirall)		Cl	OCH_2 	discontinued (1993)
lintopride (Delagrange/ Synthelabo)		Cl	OCH_3	phase II (1992)
mosapride (Dainippon)		Cl	OCH_2CH_3	preregistered (1994)
BRL-20627-A (SmithKline Beecham)		Cl	OCH_3	discontinued (1991)
P-1382 (Poli Industria)		Cl	OCH_3	preclinical (1991)

Dopamine is a precursor of noradrenaline, and it has some affinity and activity at α- and β-adrenoceptors (Shepperson *et al.*, 1982; see: Willems *et al.*, 1985). A specific neurotransmitter role for dopamine would require specific dopamine receptors to be present on the intrinsic and/or extrinsic nerves. The dopamine receptor antagonists available in the late 1970s are only moderately specific for dopamine receptors and may block 5-HT or α-adrenoceptors (Kohli & Cripe, 1979; Spedding, 1980; Cox & Ennis, 1980; see: Willems *et al.*, 1985). This lack of specificity of both dopamine and its antagonists probably accounts for much of the confusion caused by several findings argueing pro and contra implication of dopamine receptor-mediated modulation of gastrointestinal tract motility (see for a more extensive review on this subject: Willems *et al.*; 1985).

Many of the studies on the dopaminergic issue have been done on guinea-pig isolated gastrointestinal smooth muscle preparations. The isolated guinea-pig gastroduodenal preparation (Van Nueten *et al.*, 1978) exhibits spontaneous activity. In this preparation, intra-arterially administered dopamine caused relaxation which was antagonized by domperidone, clebopride and metoclopramide, in this respective rank order of potency (Schuurkes *et al.*, 1985a). Furthermore, these

compounds enhanced the amplitude of the contractions in the antrum and propagation of antral contractions over the pyloric region into the proximal duodenum (Schuurkes *et al.*, 1985a). These results led the authors to propose that dopamine D_2 receptors are present in this preparation, and that the enhanced motility is caused by blockade of these dopamine D_2 receptors. To exclude possible involvement of α-adrenoceptors, Schuurkes & Van Nueten (1981; 1984) showed that domperidone did not antagonize relaxations to intra-arterially administered noradrenaline and that relaxations to intra-arterially administered dopamine were unaffected by the α_1-adrenoceptor antagonist prazosin. Hence, involvement of α_2-adrenoceptors was not excluded.

Detailed analysis of the effects of dopamine and several adrenoceptor agonists and antagonists on stimulated and unstimulated circular muscle strips from several regions of the guinea-pig stomach, did not provide evidence for the presence of dopamine receptors in this preparation (Sahyoun *et al.*, 1982a, b, c; Costall *et al.*, 1983; 1984). Instead, it was suggested by the authors that dopamine (and domperidone) acted on α_1- and α_2-adrenoceptors (Sahyoun *et al.*, 1982a, b; Costall *et al.*, 1983; 1984). Ennis & Cox (1980) have suggested that domperidone is a competitive antagonist at α_1-adrenoceptors. Comparing receptor binding affinity data at dopamine receptors and α-adrenoceptor subtypes with data on gastric contractility and the release of acetylcholine from the guinea-pig stomach, it was suggested by Takeda *et al.* (1991) that clebopride enhanced motility by blocking peripheral dopamine D_2-receptors and α_2-adrenoceptors.

Similarly, it has been suggested that in guinea-pig isolated longitudinal muscle strips of the oesophageal junction, domperidone produced a preferential blockade of dopamine-induced relaxations (Ennis *et al.*, 1978) though later results did not support the presence of a dopaminergic system in this preparation (Cox & Ennis, 1980). In a consecutive study it was shown that there was a close correlation between the ability of a series of dopamine receptor antagonists to block α_1-adrenoceptors in the aorta and to inhibit dopamine-induced relaxations in the gut (Ennis & Cox, 1980). In the same study, the effects of a series of catecholamines on the lower oesophageal sphincter of the guinea-pig was investigated, and it was shown that domperidone blocked the effects to noradrenaline and dopamine *via* α-adrenoceptor antagonism (Ennis & Cox, 1980).

Thus, although it is difficult to explain all of the above described and other results solely by an interaction with adrenoceptors, the presence of functional specific dopamine receptors in the guinea-pig gastrointestinal tract has as yet not convincingly been demonstrated.

As clebopride and metoclopramide are both indirect stimulants of the cholinergic system, and since side effects to these drugs were observed in some patients due to central antidopaminergic actions, screening programs were initiated for compounds devoid of anti-dopaminergic properties but retaining the cholinergic actions. This resulted in the birth of the second generation benzamides, e.g. cisapride, dazopride and BRL 20627 (see Table 1.1). Cisapride and dazopride show increased potency as gastrointestinal prokinetics, whereas they have less affinity for dopamine receptors. Schuurkes *et al.* (1987) showed, both *in vitro* and *in vivo*, that there was no correlation when potency to stimulate motility vs. affinity for dopamine receptors of 5 prokinetic benzamides were studied. For example, the ratio in binding affinity for cisapride and metoclopramide at dopamine D_2 receptors is only 3, in sharp contrast to the potency ratio of almost 200 to stimulate a guinea-pig ileum preparation (Schuurkes *et al.*, 1987).

Thus, for individual compounds such as the non-benzamide domperidone and the substituted benzamides clebopride and metoclopramide, interference with dopaminergic extrinsic innervation (e.g. in the sympathetic ganglia; see Willems *et al.*, 1985) may play a role in the mechanism of action of these drugs. However, it is no longer believed that the common factor in the mechanism of prokinetic action of the substituted benzamides is dopamine D_2 receptor antagonism.

Cholinergic mechanisms

Already in 1957, Paton showed that electrical field stimulation of a longitudinal muscle-myenteric plexus preparation (LMMPP) of the guinea-pig ileum causes a contraction that is due to acetylcholine release from the enteric nerves (Paton, 1957). Metoclopramide was shown to enhance these field stimulation-induced cholinergic contractions (Fontaine & Reuse, 1972; 1973; 1979), as do cisapride, dazopride, cinitapride, renzapride and clebopride (Massingham *et al.*, 1985; Schuurkes *et al.*, 1985b; 1987; Sanger, 1987a). In the presence of either the Na^+ channel blocker tetrodotoxin (TTX) or the muscarinic cholinoceptor antagonist atropine, but not after hexamethonium (ganglionic nicotinic cholinoceptor antagonist) treatment, these contraction-enhancing effects were blocked (Massingham *et al.*, 1985; Schuurkes *et al.* 1985b; 1987) which shows their critical dependence on the postganglionic cholinergic transmission. Such an enhancement of cholinergic contractions in this preparation can be explained in several ways. Firstly, the benzamides could inhibit acetylcholinesterase activity or cause

sensitization of cholinoceptors. However, it has been shown that the benzamides did not shift the concentration-response curve to acetylcholine and methacholine on the guinea-pig ileum, whereas the acetylcholinesterase inhibitor neostigmine did (Massingham *et al.*, 1985; Schuurkes *et al.*, 1985b; 1987). Thus, inhibition of acetylcholinesterase or sensitization of cholinoceptors cannot account for the enhancement of contraction of the stimulated ileum. Secondly, the benzamides could have a direct effect on either muscarinic or nicotinic receptors. However, on the LMMPP preparation of the ileum the benzamides have no or only a small effect, while muscarinic and nicotinic cholinoceptor agonists induce large contractions (Massingham *et al.*, 1985; Schuurkes *et al.*, 1985b; 1987). Furthermore, cisapride does not have affinity for muscarinic cholinoceptors in receptor binding studies (Hasler *et al.*, 1991; Heylen *et al.*, 1992). Hence, it seems most likely that cisapride enhanced the release of acetylcholine. Indeed, in more recent studies it has been repeatedly shown that benzamides increase the amount of [^{3}H]-acetylcholine that is released upon electrical stimulation of the guinea-pig ileum LMMPP (Kilbinger *et al.*, 1982; Pfeuffer-Friederich & Kilbinger, 1984; Bou *et al.*, 1988; Taniyama *et al.*, 1991).

The responses to benzamides of the guinea-pig colon are more complex. Beani *et al.* (1970) observed metoclopramide to contract the colon through a partially TTX and atropine-sensitive but hexamethonium-insensitive pathway. In their study, they did not find metoclopramide to increase acetylcholine release (determined by bioassay) or to affect cholinesterase activity. Instead, it was shown that metoclopramide selectively sensitized contractions to exogenous acetylcholine in the presence of TTX. Hence, it was suggested that in the colon metoclopramide sensitizes the muscarinic cholinoceptors on the smooth muscle, instead of increasing acetylcholine output. As in the colon there is constant basal release of acetylcholine, the atropine and TTX-sensitive increase in tone due to metoclopramide was thus also explained *via* sensitization by the authors (Beani *et al.*, 1970). To date, a similar study on the guinea-pig colon has not been repeated, nor has it been investigated whether other benzamides could have similar sensitizing actions. In rabbit distal colon, however, cisapride has also been observed to selectively sensitize cholinergic responses mediated by postjunctional muscarinic receptors (prof. M. Tonini, personal communication). In the intact guinea-pig ileum and the taenia coli, as well as in the rabbit ileum and duodenum, metoclopramide increased tone and potentiated contractions to substance P, acetylcholine, barium ions, carbachol and nicotine (Bury & Mashford, 1976; Okwuasaba & Hamilton, 1976). According to Bury & Mashford (1976), contractions to histamine were also potentiated, at variance with the

observations of Okwuasaba & Hamilton (1976). In both studies, TTX and atropine partially or fully prevented the sensitization after metoclopramide treatment. Hence, it was suggested that all of the mentioned contractile agents act (partially) on the enteric nerves to release acetylcholine, and that metoclopramide enhances this cholinergic transmission (Bury & Mashford, 1976; Okwuasaba & Hamilton, 1976). Birtley & Baines (1973) found metoclopramide at 10 and 30 μg/ml to enhance submaximum contractions to histamine and acetylcholine in both circular and longitudinal muscle of the guinea-pig ileum. Fontaine & Reuse (1973) reported an enhancement of contractions to nicotine and acetylcholine, but not histamine, on the guinea-pig ileum longitudinal contraction. However, in both studies TTX was not tested on the metoclopramide-induced amplification responses, and therefore presynaptic effects rather than postsynaptic sensitization were not excluded.

In isolated guinea-pig gastroduodenal preparations (Van Nueten *et al.*, 1978), only metoclopramide and cisapride enhanced antroduodenal coordination of spontaneous pressure waves, while clebopride and BRL 20627 tended to; dazopride had no stimulating effect. Atropine prevented the stimulating effect of cisapride, indicating that cholinergic nerves are involved (Schuurkes *et al.*, 1985b; 1987). Cisapride and metoclopramide were also shown to enhance the spontaneous contractions in this preparation (Schuurkes *et al.*, 1985b). In isolated longitudinal muscle strips of the guinea-pig stomach, metoclopramide stimulated the spontaneous movements. This stimulation was inhibited by the muscarinic cholinoceptor blocker hyoscine but not by TTX or the ganglionic nicotinic cholinoceptor blocker hexamethonium (Hay, 1977). Na^+-action potential-independent release of acetylcholine from the nerve endings could explain these results. The release could be enhanced by metoclopramide or it could sensitize the smooth muscle muscarinic receptors. However, metoclopramide-induced enhancement of contractions to exogenous acetylcholine in this preparation were prevented by TTX and hexamethonium, suggesting that this effect depends upon ganglionic stimulation by the exogenous acetylcholine rather than a post-junctional sensitization. Hence, metoclopramide probably stimulates motility of this preparation by increasing the amount of acetylcholine that is released (Hay, 1977). In a subsequent study (Hay & Man, 1979), this hypothesis was further elaborated. Stomach strips were loaded with hemicholinium-3 to diminish acetylcholine content. In such strips, the contractile activity to metoclopramide was prevented, providing further support for a neuronal acetylcholine-release enhancing action of metoclopramide in this tissue (Hay & Man, 1979).

It is now fully accepted, that nerves contain usually more than one neurotransmitter (see for review on co-transmission: Burnstock, 1990). In enteric nerves of the guinea-pig it has been shown with immunostaining techniques that motor neurons that stain for choline acetyltransferase (a marker for acetylcholine) usually contain substance P-immunoreactive material as well (Furness *et al.*, 1988). This suggests that both neurotransmitters are released upon nerve stimulation, though the ratio of the released amounts might depend upon the stimulation frequency (Furness *et al.*, 1988; Burnstock 1990). It is thus conceivable that benzamide-induced facilitation of cholinergic transmission through an increase in acetylcholine release would also lead to an increase in substance P release. Furthermore, benzamides could interfere with the release of other non-cholinergic substances that are released from non-cholinergic neurons. These non-adrenergic, non-cholinergic (NANC) neurotransmitters are believed to play an important role in gut motility (see Bingham & Andrews, 1992). In the guinea-pig colon, it has been demonstrated that cisapride enhances the field-stimulation-induced relaxation under NANC conditions (Schuurkes, 1992), suggesting enhanced release of an inhibitory neurotransmitter. In the presence of atropine, cisapride facilitated the contractions in the gastric corpus and antrum in response to submaximal vagal efferent stimulation of the anaesthetized ferret (Bingham & Andrews, 1987; 1992). Cisapride and other benzamides have been shown to facilitate 5-HT-evoked purinergic inhibitory transmission in the guinea-pig proximal colon (Briejer *et al.*, 1994). In circular smooth muscle tissue of the guinea-pig ileum renzapride facilitated the field stimulation-evoked release of non-cholinergic inhibitory (purinergic) and excitatory transmitters (King & Sanger, 1992). These results strongly suggest that not only facilitation of cholinergic transmission, but also facilitation of non-cholinergic transmission might contribute to the motility-stimulating effects of the benzamides. Future studies should address this question in more detail.

Though it was becoming more and more evident that benzamides facilitate cholinergic transmission, it was not known *via* which receptor type(s) this could be established. Schuurkes *et al.* (1988) investigated whether cisapride could enhance acetylcholine release in guinea-pig ileum LMMPP by antagonizing presynaptic muscarinic M_1 cholinoceptors. Stimulation of M_1 cholinoceptors is known to cause inhibition of acetylcholine release (autoreceptors) (North *et al.*, 1985; Buckley & Burnstock, 1986). However, cisapride could not antagonize the inhibition of field stimulation-induced twitch contractions caused by the M_1 cholinoceptor agonist McN-A-343, while the M_1 cholinoceptor antagonist pirenzepine did. Furthermore, enhancement of twitch contractions induced by

cisapride on the ileum was not affected by the presence of pirenzepine, nor did pirenzepine itself cause stimulation. Hence, it was concluded that cisapride does not interact with M_1 cholinoceptors to stimulate cholinergic transmission (Schuurkes *et al.*, 1988).

Summarizing, it has been established that the most important property of substituted benzamides is their ability to facilitate cholinergic neurotransmission. This is done by increasing the amount of acetylcholine that is released per stimulus, but in some cases results have also suggested that postjunctional cholinoceptors are sensitized. Facilitation of non-cholinergic neurotransmission might also play a role. The former and latter hypotheses deserve to be studied in more detail.

Interactions with 5-HT_3 receptors

Already in 1970, Bianchi *et al.* reported that metoclopramide inhibited contractions to 5-hydroxytryptamine (5-HT; serotonin) on the guinea-pig colon. Similar observations have been made on other guinea-pig gastrointestinal preparations as well (Fontaine & Reuse, 1973; Birtley & Baines, 1973). At that time it was still presumed that two types of 5-HT receptors existed in the guinea-pig gut, M and D receptors. In 1957, Gaddum and Picarelli had shown that contractions to 5-HT could be partially inhibited by morphine (M-receptors) and dibenzyline (= phenoxybenzamine; D receptors) (Gaddum & Picarelli, 1957). However neither compound is a specific 5-HT receptor antagonist. Dibenzyline is an alkylating agent, blocking smooth muscle responses to many compounds (Boyd *et al.*, 1963; Day & Vane, 1963), and morphine inhibits acetylcholine release by stimulating presynaptic inhibitory opioid receptors (Paton, 1957; Schultz & Cartwright, 1974). Due to a lack of selective 5-HT receptor antagonists, no great progress was made in the 1960s and 1970s in the classification and identification of 5-HT receptor types. In the 1970s, the radioligand binding technique was developed, and it was found in 1979 that 5-HT binds at two different binding sites in the central nervous system for which lysergic acid diethylamide has a high affinity. These binding sites were designated 5-HT_1 and 5-HT_2 (Peroutka & Snyder, 1979). Subsequent studies showed that 5-HT antagonists with affinity for the 5-HT_2 site showed a good correlation with D receptors measured on functional studies in vascular and gastrointestinal smooth muscle (Humphrey *et al.*, 1982; Engel *et al.*, 1984; 1985; Maayani *et al.*, 1984).

Cocaine was found to be a relatively selective antagonist at M receptors on sympathetic nerve endings in the rabbit heart (Fozard *et al.*, 1979). From cocaine, more potent and selective M receptor blocking drugs were derived, e.g. MDL 72222 (Fozard, 1984) and ICS 205-930 (= tropisetron; Richardson *et al.*, 1985). With these compounds, it could be confirmed that M receptors do not fall into the 5-HT_1 and 5-HT_2 category (see Table 1.2). The confusion in the 5-HT receptor classification field was greatly abolished by Bradley *et al.* by publishing their proposals for 5-HT receptor classification (Bradley *et al.*, 1986; see Table 1.2). The M receptor was now called 5-HT_3. Within the 5-HT_1 subtype there was evidence for heterogeneity, but lack of selective tools did not allow further subclassification at the time.

Table 1.2 Ligands for the classification of 5-HT receptor types according to Bradley *et al.* (1986)

	5-HT_1-like*	5-HT_2	5-HT_3
former name	-	D	M
"selective" antagonists known in 1986	methiothepin methysergide	methiothepin methysergide ketanserin spiperone	MDL 72222 tropisetron (-)-cocaine
"selective" agonist	5-CT	(+)-s-α-methyl-5-HT	2-methyl-5-HT phenylbiguanide

5-CT = 5-carboxamidotryptamine;*evidence for heterogeneity within this receptor type.

As stated before, it was known that metoclopramide antagonizes neurogenic contractions to 5-HT in the guinea-pig gut (Bianchi *et al.*, 1970; Fontaine & Reuse, 1973; Birtley & Baines, 1973), which suggests an interaction with 5-HT_3 receptors. In strips of guinea-pig stomach, metoclopramide as well as the 5-HT_3 receptor antagonists tropisetron and MDL 72222 enhanced field stimulation-induced contractions (Buchheit *et al.*, 1985a). Again these results suggest that metoclopramide is an antagonist at 5-HT_3 receptors. In the same year, several groups suggested that 5-HT acts upon two different neuronal 5-HT receptors in the guinea-pig ileum to facilitate field stimulation-induced acetylcholine release (Kilbinger & Pfeuffer-Friederich, 1985; Fozard, 1985). It was also suggested that in this ileum preparation 5-HT causes contractions *via* two different 5-HT receptors (Buchheit *et al.*, 1985b). Renzapride (BRL 24924) was observed to enhance field stimulation-induced twitch contractions more potently but similarly to metoclo-

pramide in the ileum, which could be prevented by high concentrations of 5-HT but not by a 5-HT_3 receptor blocking concentration of tropisetron. Renzapride also antagonized 5-HT-evoked, cholinergically mediated, contractions on this preparation (Sanger, 1987a). Also cisapride has been shown to enhance the cholinergic field-stimulation-induced contractions both in the absence and presence of a 5-HT_3 receptor blocking concentration of tropisetron (Schuurkes & Van Nueten, 1988). In the guinea-pig ileum, contraction and facilitation of cholinergic transmission to metoclopramide was prevented by desensitization with 5-HT (Kilbinger *et al.*, 1982). Sanger, as well, showed that on the guinea-pig ileum preparation, at low concentrations metoclopramide acted upon a receptor susceptible to desensitization by 5-HT to induce facilitation of cholinergic transmission (at higher concentrations of metoclopramide 5-HT could not produce desensitization, suggesting an additional mechanism) (Sanger, 1987b). Also for cisapride and cinitapride, actions on the guinea-pig ileum and colon similar to those described above have been observed (Pfeuffer-Friederich & Kilbinger, 1984; Schuurkes *et al.*, 1985b; Massingham *et al.*, 1985). These observations indicate that two different 5-HT receptor types are present on the myenteric neurons, that both facilitate cholinergic transmission. One is the 5-HT_3 receptor, at which most benzamides are moderate affinity antagonists (see Table 1.3). The other 5-HT receptor, at which the benzamides are agonists, was unknown.

Much effort has been made to elucidate whether the 5-HT_3 receptor blocking property of benzamides can explain their motility-stimulating action. The development of the potent and selective non-benzamide 5-HT_3 receptor antagonists GR 38032F (ondansetron; Brittain *et al.*, 1987), tropisetron (Richardson *et al.*, 1985) and BRL 43694 (granisetron; Sanger & Nelson, 1989) (see Table 1.3) gave an impetus to answer this question. Motility experiments with 5-HT_3 receptor antagonists have been performed in the guinea-pig, rat, dog, and to a lesser extent in man.

Guinea-pig

Facilitation of cholinergic transmission could explain the gastrointestinal motility-stimulating properties *in vivo* of the benzamides, as acetylcholine is the main excitatory neurotransmitter involved in both nerve-nerve and nerve-smooth muscle communication. Stimulation of neuronal 5-HT_3 receptors causes facilitation of cholinergic transmission in guinea-pig gastrointestinal preparations, as described above. Thus, the 5-HT_3 receptor antagonist properties of most benzamides is difficult to reconcile with their prokinetic properties, if it is supposed that facilitation of cholinergic transmission is their key property.

Table 1.3 Potency of 5-HT_3 receptor antagonists and prokinetic benzamides in several established *in vitro* and *in vivo* 5-HT_3 receptor models and in *in vivo* gastrointestinal motility models.

compound	[1]Radioligand binding 5-HT_3 sites rat cortex K_i (nM)	[2]Depolarization isolated rat vagus nerve to 5-HT K_b (nM)	[3]Bezold-Jarisch reflex anaesthetized rat ED_{50} (µg/kg i.v.)	[3]Gastric emptying solid beads rat ED_{50} (µg/kg i.p.)
5-HT_3 antagonists				
ondansetron	0.4-4.8	2.5	5.23	-
granisetron	0.1-2.7	0.2	0.70	-
tropisetron	0.2-3.2	0.01	0.6	9.7
benzamides				
metoclopramide	120-457	251	1398	1054
cisapride	135	-	2022	509
renzapride	-	-	12.4	26.8
zacopride	0.1-2.0	0.1	0.4	4.5

compound	[4]Gastric emptying solid meal dog ED_{50} (µg/kg i.g.)	[5]Antiemetic activity cisplatin-induced emesis dog ED_{50} (mg/kg i.v.)	[3]Heidenhain pouch gastric motility dog dose (µg/kg i.v.) resp. pos/tested
5-HT_3 antagonists			
ondansetron	-	0.008	-
granisetron	-	-	-
tropisetron	-	0.01	100: 0/3
benzamides			
metoclopramide	-	0.6	100: 5/6
cisapride	200	0.6	100: 3/4
renzapride	100	0.03	30: 3/3
zacopride	-	[6]0.002	30: 5/5

References: [1]Kilpatrick *et al.*, 1990; [2]Butler *et al.* 1990; [3]Schiavone *et al.*, 1990; [4]Gullikson *et al.*, 1993; [5]Gullikson *et al.*, 1991; [6]Cohen *et al.*, 1989.

It has been shown that metoclopramide, but also tropisetron and ondansetron, increase the rate of gastric emptying of radio-opaque beads in conscious guinea-pigs (Buchheit *et al.*, 1985a; Costall *et al.*, 1987). Costall *et al.* (1986) investigated whether a possible central 5-HT_3 receptor mechanism could be involved in gastroprokinetic effects. Tropisetron was injected directly into the hypothalamic region of conscious guinea-pigs, which caused an increase of the rate of gastric emptying. 5-HT and 2-methyl-5-HT had decreased gastric emptying,

which could be antagonized by tropisetron injection in the hypothalamus (Costall *et al.* 1986). Hence, extra-enteric 5-HT_3 receptors might play a role in the regulation of stomach motility in the guinea-pig. In conscious guinea-pigs, ondansetron, tropisetron and granisetron delayed fecal pellet transit. In contrast to these *in vivo* experiments, ondansetron was without activity on spontaneous pellet propulsion in the isolated colon, though granisetron and tropisetron did inhibit transit (Sanger *et al.*, 1991). The conflicting results obtained with ondansetron vs. granisetron and tropisetron might reflect differences in ability to reach the receptor site. Wade & Gershon (1991) also found that, in a similar preparation, 5-HT_3 receptor blocking concentrations of tropisetron, granisetron and zacopride did not affect colonic propulsion, while renzapride inhibited propulsion. In the Trendelenburg (1917) setup of the guinea-pig ileum, 5-HT_3 receptor blocking concentrations of tropisetron, ondansetron and granisetron did not affect the peristaltic reflex, while benzamides like cisapride, metoclopramide, renzapride and others facilitated the reflex (Craig & Clarke, 1991; Buchheit & Buhl, 1991; 1993; Wade & Gershon, 1991). In the Trendelenburg experiment, zacopride had a pEC_{50} value of 5.7 (Buchheit & Buhl, 1991), while it has nanomolar affinity for 5-HT_3 receptors, indicative for a non-5-HT_3 receptor mechanism. From the above described results it can be concluded that the prokinetic benzamides are not likely to stimulate gastrointestinal motility in the guinea-pig by blocking 5-HT_3 receptors. 5-HT_3 receptor blockade might however contribute to the effects on gastric emptying in this species.

Rat

In the rat, stomach-to-caecum transit was delayed by subcutaneously administered ondansetron and granisetron. However, lipid-delayed transit was reversed by ondansetron and granisetron (Brown *et al.*, 1993b). Ondansetron enhanced the action of a protein-rich solution in delaying gastric emptying in conscious fistula rats, but it did not affect emptying of isotonic or hypertonic saline, acid or FOY-305, which delays gastric emptying by release of cholecystokinin (Forster & Dockray, 1990). These data show that drug effects on motility may greatly depend upon the experimental conditions, especially the type of meal. The effects of the benzamides cisapride, zacopride, metoclopramide and renzapride, as well as the 5-HT_3 receptor antagonist tropisetron, were tested on the rate of gastric emptying of 20 Amberlite® beads in conscious rats (see Table 1.3). All tested compounds accelerated gastric emptying with potencies that correlated well with the potencies for inhibiting the Bezold-Jarisch reflex (5-HT-induced bradycardia in the anaesthetized rat, an established *in vivo* 5-HT_3 model) (Schiavone *et al.*, 1990).

Hence, 5-HT_3 receptor antagonism could well account for the stimulating effects of benzamides on gastric emptying in this species. Cohen *et al.* (1990), however, showed that zacopride, but not the potent and selective 5-HT_3 receptor antagonist LY277359, accelerated gastric emptying of a complex meal. Despite the fact that only 2 compounds were tested, these observations plea against a 5-HT_3 antagonistic mechanism in the stimulating effect of gastric emptying in the rat. Colonic transit as measured by counting fecal pellet output in conscious rats, was not affected by ondansetron and granisetron p.o. (Kadowaki *et al.*, 1993), but effects of benzamides on colonic transit are not available. Hence, in the rat 5-HT_3 receptor blockade seems an important feature of the benzamides to stimulate gastric motility, though it is likely that other properties are also important.

Dog

In conscious dogs equipped with strain gauge force transducers, tropisetron slightly reduced gastroduodenal motility, while cisapride enhanced it (even in the presence of tropisetron). Cisapride accelerated gastric emptying of a liquid meal, but ondansetron did not affect gastric emptying (Schuurkes & Van Nueten, 1988). Gullikson *et al.* (1991) tested a number of benzamides and 5-HT_3 receptor antagonists in the conscious dog. All drugs tested were 5-HT_3 receptor antagonists as they blocked cisplatin-induced emesis (see: Andrews *et al.*, 1988) with relative potencies of granisetron = tropisetron > renzapride > cisapride = metoclopramide. In contrast, the order of potency for stimulation of antral motility was: renzapride = cisapride > metoclopramide > tropisetron = granisetron (see Table 1.3). Renzapride and cisapride stimulated jejunal myoelectric activity but tropisetron was not active. Renzapride and cisapride also increased the rate of emptying of solid and liquid meals, but tropisetron only enhanced liquid emptying whereas granisetron had no effect at all (Gullikson *et al.*, 1991). In conscious dogs chronically implanted with force transducers, ondansetron, granisetron and tropisetron did not affect digestive gastrointestinal motility, whereas renzapride stimulated digestive motility from stomach to colon (Yoshida *et al.*, 1991b). Hence, in the dog the prokinetic activity of benzamides seems unrelated to 5-HT_3 receptor blockade.

Human

In man, ondansetron, did not accelerate gastric emptying or small bowel transit, while large bowel transit was slowed (Gore *et al.*, 1989; 1990; Talley *et al.*, 1989; 1990; Nielsen *et al.*, 1990). Tropisetron has been found to slightly increase and reduce gastric emptying in man, depending on the dose (Akkermans *et al.*,

1988; Stacher *et al.*, 1990) and to produce mild constipation and colonic hypermotility (Stacher *et al.*, 1989). Furthermore, tropisetron has been observed to speed up mouth-to-caecum transit in man (Meleagros *et al.*, 1987). Recently, ondansetron, given intravenously, has been found to inhibit the initiation of gastric phase-3 activity of the interdigestive cyclical pattern of motor activity (migrating motor complex) in humans (Wilmer *et al.*, 1993), suggesting 5-HT_3 receptor involvement. It is however difficult to extrapolate the significance of these findings to gastroprokinetic effects, as the latter take place in the digestive state, in which motility patterns totally different from those in the fed state (when prokinetics ought to exert their effect) play a role. Nevertheless, these observations suggest that 5-HT_3 receptors do play a role in the motility of the intestines in humans. However, benzamides such as cisapride stimulate gastric and bowel motility in healthy humans as well as in patients with a variety of motility disorders (see: Wiseman & Faulds, 1994). Lack of consequent stimulating effects on motility of 5-HT_3 receptor antagonists render the possibility that the benzamides stimulate motility in man solely by antagonising these 5-HT_3 receptors rather unlikely.

Interactions with 5-HT_4 receptors

In the mouse colliculi neurons and in guinea-pig hippocampal membranes, Shenker *et al.* (1987) and subsequently Dumuis *et al.* (1988) identified a high affinity 5-HT receptor which was positively coupled to adenylate cyclase. The pharmacological and radioligand binding profile of the tested agonists and antagonists differed completely from the profile for the 5-HT_1-like, 5-HT_2 and 5-HT_3 receptors (Table 1.2). Hence, this receptor was proposed to be designated 5-HT_4 (Dumuis *et al.*, 1988) In subsequent studies, it was shown that the gastrointestinal prokinetic benzamide derivatives, such as cisapride, renzapride, zacopride, BRL 20627 and metoclopramide were agonists at this 5-HT_4 receptor, in this respective rank order of potency (Dumuis *et al.*, 1989a; b; Bockaert *et al.*, 1990). The only antagonist available at the time appeared to be tropisetron, be it at micromolar concentrations (pK_i = 6.0-6.3 at 5-HT_4 receptors versus 8-10 at 5-HT_3 receptors) (Dumuis *et al.*, 1988; 1989a; b).

Guinea-pig

In most tissues, the neurogenic M receptors as defined by Gaddum and Picarelli (1957) were shown to correspond to the 5-HT_3 receptors, as proposed by

Bradley *et al.* (1986). However, observations made on the guinea-pig ileum have led to considerable debate as to whether the M receptor and 5-HT_3 receptor are the same entity. As discussed above, it was suggested that two types of neuronal 5-HT receptor with similar action exist in this tissue (Buchheit *et al.*, 1985b; Kilbinger & Pfeuffer-Friederich, 1985; Fozard, 1985). M-receptors were originally defined to account for the neuronally-mediated effects to 5-HT in the ileum. Hence, the question which of these two receptors corresponded to the M-receptor gave rise to confusion. The discovery of the 5-HT_4 receptor solved this issue (see for more extensive review on this issue: Hoyer, 1990). The high and low affinity phase of the biphasic concentration-response curve to 5-HT on the ileum, and also on the colon, were shown to be mediated by 5-HT_4 and 5-HT_3 receptors, respectively (Eglen *et al.*, 1990; Briejer *et al.*, 1993a). Tropisetron antagonized the enhancement of the electrically-induced twitch contractions due to 5-HT, as well as to cisapride, zacopride and renzapride with micromolar affinity (Craig & Clarke 1990). Elaborating the observation originally made by Fozard (1985), who observed that with 5-methoxytryptamine (5-MeOT) two neurogenic receptors on the guinea-pig ileum could be discriminated, Craig *et al.* (1990) showed that 5-HT_3 and 5-HT_4 receptors could be selectively desensitized by 2-Me-5-HT (a 5-HT_3 receptor agonist, see Table 1.2) and 5-MeOT, respectively. The presence of 5-HT_4 receptors mediating the release of acetylcholine from the ileal enteric nerves has been confirmed in several studies with different techniques, e.g. determination of [^{3}H]acetylcholine release (Kilbinger & Wolf, 1992) and mutual desensitization of cisapride, 5-HT and 5-MeOT (Meulemans & Schuurkes, 1992). The development of novel, more selective and potent 5-HT_4 receptor antagonists (SDZ 205-557, DAU 6285) and agonists (BIMU 1, BIMU 8) allowed further identification of 5-HT_4 receptors in this preparation (Buchheit *et al.*, 1992; Rizzi *et al.*, 1992). Tonini and co-workers showed that also on the enteric nerves innervating the circular smooth muscle of the guinea-pig ileum, 5-HT_4 receptors are present. Also in this preparation, cisapride induced an increase in field stimulation-induced cholinergic twitch contractions (Tonini *et al.*, 1992).

The 5-HT_4 receptor was soon identified in other tissues. The remaining contractions to 5-HT after 5-HT_1, 5-HT_2 and 5-HT_3 blockade on the guinea-pig colon were shown to be sensitive to micromolar concentrations of tropisetron, as were those to cisapride, renzapride, metoclopramide and zacopride. The contractions were strongly, but not fully inhibited by atropine and abolished by TTX, demonstrating involvement of cholinergic nerves (Elswood *et al.*, 1991). The fact that in the presence of atropine still a part of the contraction remained might indicate that also a non-cholinergic transmitter (substance P) is involved, as has

been shown after 5-HT_3 and 5-HT_4 receptor stimulation in the ileum (Fox & Morton, 1991; Ramirez *et al.*, 1994; see discussion above on co-transmission). Hence it was concluded that 5-HT_4 receptors are present on the guinea-pig colon enteric nerves (Elswood *et al.*, 1991). These observations have later been confirmed (Briejer *et al.*, 1993a; b; Gale *et al.*, 1994). In LMMPP of guinea-pig distal colon, 5-HT_4 receptors have also been shown to be present. They seem very effectively coupled to their second messenger system, as reflected by a high pEC_{50} value of 9.2 (Wardle & Sanger, 1993; Wardle *et al.*, 1994). The same workers recently also showed 5-HT, 5-MeOT, renzapride and zacopride to induce tropisetron and SDZ 205-557-sensitive contractions of the circular muscle preparation of the distal colon. The location on cholinergic nerves of these 5-HT_4 receptors however was not verified (Ellis *et al.*, 1994). On isolated circular smooth muscle strips from the guinea-pig stomach fundus and antrum, 5-HT, metoclopramide and renzapride enhanced electrical field stimulation-induced contractions by 300-400 %. These effects could be antagonized with SDZ 205-930 but not by tropisetron in a 5-HT_3 receptor blocking concentration. 2-Me-5-HT also caused stimulation, but this effect was insensitive to SDZ 205-557. The authors concluded that despite the presence of 5-HT_3 receptors in their preparation, these effects were mediated predominantly by 5-HT_4 receptors (Buchheit & Bertholet, 1992). Hence, along the guinea-pig gastrointestinal tract, 5-HT_4 receptors, for which benzamides are agonists, are abundantly present on the enteric nerves.

Rat

Crossing the species border, very different results have been obtained. In contrast to the guinea-pig, 5-HT_4 receptors in the rat gastrointestinal tract generally appear to be located on the smooth muscle, mediating relaxation. Like in the guinea-pig, rat 5-HT_4 receptors have been found to be positively coupled to adenylate cyclase, which is in agreement with a direct smooth muscle relaxation (Ford *et al.*, 1992; Moummi *et al.*, 1992). On intact segments of the rat oesophagus, the benzamides were partial 5-HT_4 receptor agonists with the following rank order of potency: renzapride > R,S-zacopride > metoclopramide = cisapride (Reeves *et al.*, 1991a). However, in tunica muscularis mucosae preparations of the oesophagus, the rank order of potency was cisapride > S-zacopride = renzapride > R,S-zacopride > R-zacopride (Baxter *et al.*, 1991). These differences might be explained either to be caused by diffusion difficulties of cisapride to the 5-HT_4 receptor sites, or by an additional mechanism in the intact preparation. In the ileum, 5-HT_4 receptor stimulation also caused relaxation *via* an atropine-insensitive mechanism, suggesting also a smooth muscle location of the 5-HT_4 receptors

(Taludhar *et al.*, 1991; 1992). In subsequent studies it was confirmed that these 5-HT receptors were indeed 5-HT_4 receptors (Taludhar *et al.*, 1994a; b). Facilitation of cholinergic transmission as the mechanism for prokinetic action of the benzamides is difficult to reconcile with smooth muscle 5-HT_4 receptor activation inducing relaxation. Therefore, *in vivo* studies in the rat might provide new insights as to their mechanism of action. In rats fed with a methylcellulose test meal containing radioactive microspheres, metoclopramide, renzapride and R,S-zacopride dose-dependently stimulated cholecystokinin-delayed gastric emptying. The increase in gastric emptying could not be mimicked or inhibited by 5-HT_1, 5-HT_2, 5-HT_3 or dopamine D_2-receptor antagonists, but 5-HT_4 receptor blocking doses of tropisetron or the potent 5-HT_4 receptor antagonist GR125487 prevented the stimulation after metoclopramide administration (Reeves *et al.*, 1991b; Gale *et al.*, 1993). Unfortunately, no *in vitro* study on the location -either neuronal or muscular- of 5-HT_4 receptors in the stomach has been published for comparison. In conscious rats equipped with a duodenal cannula, neither zacopride nor cisapride could stimulate small intestinal transit of a methylcellulose test meal containing radioactive microspheres after slowing of transit by the α_2-adrenoceptor agonist p-aminoclonidine (Clayton & Gale, 1994). This observation is in agreement with the smooth muscle localization of 5-HT_4 receptors on the smooth muscle, and a consequent lack of stimulation of cholinergic transmission. In anaesthetized rats, gastric emptying of a radiolabelled liquid was accelerated by intra-arterial 5-HT and by R-zacopride, which is a potent 5-HT_3 receptor antagonist but only a low potency 5-HT_4 receptor agonist (see Tables 1.3 and 1.4). Moreover, the benzamide SC-53116, which has been reported to be a potent 5-HT_4 receptor agonist but only a moderate potency 5-HT_3 receptor antagonist (Flynn *et al.*, 1992; Gullikson *et al.*, 1993; see Table 1.4) had a much less pronounced effect on gastric emptying (Valdovinos *et al.*, 1993). These observations are in agreement with the previously drawn conclusion that 5-HT_3 receptor antagonism rather than 5-HT_4 receptor agonism could be important in benzamide-induced acceleration of gastric emptying in the rat. However, the fact that only one dose of the drugs was tested in this study and no other 5-HT_3 or 5-HT_4 receptor (ant)agonists were used limits the strength of this conclusion. More *in vitro* and *in vivo* studies in the rat with benzamides and non-benzamide 5-HT_3 and 5-HT_4 receptor antagonists to investigate how and to what extent different benzamides exert their prokinetic effects would be welcomed.

Dog

Another species which has been studied with respect to benzamides and gastrointestinal motility, is the dog. *In vivo*, cisapride and the 5-HT_4 receptor agonist BIMU 1 dose-dependently increased gastric motility as assessed in the Heidenhain pouch (a denervated gastric pouch) in the conscious dog. These stimulating effects could not be inhibited by ondansetron, suggesting an action on 5-HT_4 rather than on 5-HT_3 receptors (Rizzi *et al.*, 1994). In a similar experiment, it was shown that the 5-HT-induced stimulation of gastric motility could be inhibited by the non-benzamide 5-HT_4 receptor antagonist SB 204070 (Bingham *et al.*, 1993). Cisapride and BIMU 1, but not ondansetron, accelerated gastric emptying of a liquid meal in the conscious dog, and this effect could be blocked by the relatively selective non-benzamide 5-HT_4 receptor antagonist DAU 6285 (Rizzi *et al.*, 1994). In similar experiments, gastric emptying of a liquid meal containing oleic acid to delay emptying, was shown to be accelerated by cisapride, metoclopramide and clebopride (in this respective order of potency) (Schuurkes *et al.*, 1987). α_2-Adrenoceptor agonist-induced delayed gastric emptying of liquid and solid meals was shown to be enhanced by cisapride, renzapride, S-zacopride and the benzamide 5-HT_4 receptor agonist SC-49518 (see Table 1.1) with about equal potency (SC-49518 has only micromolar affinity at 5-HT_3 receptors) (Gullikson *et al.*, 1992; 1993). In fasted dogs chronically implanted with force transducers or electrodes, cisapride, renzapride, R- and S-zacopride and SC-49518 also enhanced antral contractile and myoelectric activity (Gullikson *et al.*, 1992; 1993). In a similar study on dogs equipped with strain gauges, it was found that cisapride, mosapride (AS-4370; see Table 1.1) and less potently metoclopramide stimulated antral and duodenal motor activity, whereas cisapride also stimulated colonic motility (Yoshida *et al.*, 1991a). These effects of mosapride could not be antagonized by 5-HT_3 receptor-blocking doses of tropisetron, but they were sensitive to desensitization by continuous infusion of 5-HT, as were the stimulating effects to cisapride and metoclopramide (Yoshida *et al.*, 1991a). The 5-HT_4 receptor antagonist SDZ 205-557 (1.25 mg/kg s.c.) inhibited the stimulation to cisapride (1.25 mg/kg p.o.) of antropyloric motility in conscious dogs, but SDZ 205-557 *per se* had no effect (unpublished observations). Taken together, these observations obtained in conscious dogs suggest that a 5-HT_4 receptor mechanism indeed is involved in the prokinetic action of the benzamides.

Cisapride as well as 5-HT have been shown to enhance electrical field stimulation-induced cholinergic contractions of longitudinal muscle strips of the dog gastric antrum. Though it was suggested that both substances acted *via* different receptors, involvement of 5-HT_4 receptor could not be shown (De Ridder &

Schuurkes, 1993). However, this evidence was merely based upon the use of the low potency non-selective tropisetron. Recently the selective and highly potent 5-HT_4 receptor antagonist SB 204070 has been tested in the same preparation, and it was found that the effects to 5-HT, but not those to cisapride could be inhibited (W.E. De Ridder, personal communication), suggesting that cisapride acts through a mechanism other than 5-HT_4 receptors in this preparation. These results with longitudinal muscle strips of the gastric antrum (De Ridder & Schuurkes; 1993), suggesting that 5-HT_4 receptors are not involved in the effects of cisapride, may have only little relevance for gastric motility effects *in vivo*.

Human

Only few *in vitro* studies with human tissues have been conducted with respect to benzamides and 5-HT. In circular muscle strips from the fundus, corpus and antrum electrical field stimulation caused cholinergic contractions, which were enhanced by cisapride, 5-HT and 5-MeOT. These compounds antagonized each others action (mutual desensitization), and tropisetron in a 5-HT_4 receptor blocking concentration was an antagonist (Schuurkes *et al.*, 1991). Renzapride (0.0028-28 μM) enhanced field stimulation-induced cholinergic contractions, but not the non-cholinergic relaxations, of longitudinal muscle strips of the stomach. Only at high concentrations (28 and 280 μM) renzapride enhanced contractions to exogenous acetylcholine (Burke & Sanger, 1988). Metoclopramide up to 100 μg/ml (= 0.33 mM) had no effect on stomach strips, but from 1 μg/ml (= 3.3 μM) onwards it sensitized contractions to exogenous acetylcholine (Eisner, 1968). Hence, in human stomach the benzamides facilitate cholinergic transmission, probably due to an increase in acetylcholine release. Sensitisation of postsynaptic cholinoceptors cannot be excluded, but this effect probably only contributes to facilitation of cholinergic transmission at very high, non-therapeutical, concentrations of benzamide.

Eisner (1968) reported that metoclopramide had no effect on longitudinal muscle strips of the colon. Metoclopramide (0.1-10 μg = 0.33-33 μM) sensitized contractions to acetylcholine on longitudinal strips (Eisner, 1968). Burleigh (1977) found that 5-HT induced contraction *via* a methysergide (5-$HT_{1/2}$ receptor antagonist)-sensitive mechanism, and also relaxation of longitudinal muscle strips of the colon. On the similar preparation, Hillier *et al.* (1994) showed that inhibitory 5-HT_1-like receptors are present on the longitudinal smooth muscle.

Metoclopramide (0.1-10 μg = 0.33-33 μM) caused atropine-insensitive contraction of circular muscle strips of the colon (Eisner, 1968). On such strips contracted with carbachol, 5-HT induced a relaxation which was partially inhibited by TTX

but not by methysergide (Burleigh, 1977). Tam and co-workers (1994) observed on inter-taenial circular muscle strips of the isolated human colon, that 5-HT and 5-MeOT, concentration-dependently inhibited spontaneous activity. This effect was slightly attenuated by TTX, and it was mimicked by renzapride, zacopride, metoclopramide and cisapride (in this respective rank order of potency, though the irregular shape of the curves made a reliable estimate of potency difficult). Furthermore, tropisetron was an antagonist to the effects of 5-MeOT and 5-HT with an estimated pK_b of 6, corresponding to its affinity for 5-HT_4 rather than for 5-HT_3 receptors (Tam *et al.*, 1994). In a consecutive study, these autors showed that the 5-HT_4 receptor agonists BIMU 1 and BIMU 8 also caused inhibition of spontaneous activity, and that the 5-HT_4 receptor antagonists DAU 6285 and GR 113808 were antagonists (Hillier *et al.*, 1994). On histamine-precontracted circular smooth muscle strips from human sigmoid colon, 5-HT induced relaxations that were insensitive to TTX and atropine, but which were inhibited by the 5-HT_4 receptor antagonist SB 204070 (Meulemans *et al.*, 1995). These results suggest that inhibitory 5-HT_4 receptors are present on the circular smooth muscle of the human colon. The contractile effect to metoclopramide on circular muscle, as observed by Eisner (1968), might be caused *via* other mechanisms.

In order to evaluate whether cisapride can facilitate cholinergic transmission by causing an increase of acetylcholine release in the human colon, Burleigh & Trout (1985) measured release of [^{3}H]-acetylcholine in sigmoid taenia coli. Cisapride did not affect basal or field stimulation-induced release of acetylcholine (Burleigh & Trout, 1985). On field stimulation-induced cholinergic contractions of taenia strips, renzapride had no effect up to 282 μM. Hence, these observations with cisapride and renzapride are consistent with 5-HT_4 receptors being located on the smooth muscle instead of on cholinergic nerves in the colon.

Cisapride has been reported to stimulate colonic motility as it is effective in mildly constipated patients (see: Longo & Vernava, 1993). Altaparmakov (1985) studied the effects of cisapride on descending colonic and sigmoid motility, as assessed with extracellular electrodes, in healthy volunteers and chronically constipated patients (randomized design, double blind, placebo controlled). It was observed that in both groups propulsive motility was enhanced, an effect which was not affected by i.v. injection of atropine. These results led the author to conclude that the stimulation of colonic motility by cisapride does not depend upon cholinergic pathways (Altaparmakov, 1985).

Summarizing, the benzamides are likely to enhance stomach motility by enhancing cholinergic transmission, possibly by stimulation of 5-HT_4 receptors. On the colon, the benzamides act as agonists on the inhibitory circular smooth

muscle 5-HT_4 receptors. Seemingly in contradiction to this, benzamides have moderatly stimulating effects on lower gut motility. Cholinergic transmission is not enhanced by relevant concentrations of benzamide. It is not clear how these phenomena relate to each other.

Electrophysiology of 5-HT and benzamides

The electrophysiology of the myenteric neurons has been explored in detail only in the last two decades. Single neurons in isolated myenteric ganglia (usually dissected from the guinea-pig small intestine) can be impaled with microelectrodes to measure membrane potentials and conductance. Thus, despite of the many different types of neuron that can be distinguished on an immunohistochemical basis, only 4 types of neuron can be distinguished electrophysiologically to occur in the enteric nervous system (see Bornstein *et al.*, 1994; Wood, 1994). With respect to the effects of 5-HT on myenteric neurons, only two types are important. S-Neurons (also called type I) respond with multiple spike discharges upon a depolarizing stimulus, while AH-neurons (also called type II) only show one or two spike discharges upon a depolarizing stimulus, and show a prolonged after-hyperpolarization (for more details, see Galligan & North, 1989; Gershon & Wade, 1993; Bornstein *et al.*, 1994; Wood, 1994). Three types of direct response can be observed after application of 5-HT, mediated by respectively 5-HT_3, 5-HT_{1A} and 5-HT_{1P} receptors (Nemeth *et al.*, 1985; Mawe *et al.*, 1986; see Galligan & North, 1989).

Interactions with 5-HT_3 receptors

In S/type I as well as in AH/type II neurons, 5-HT can induce a transient fast depolarization prone to rapid desensitization, associated with spike discharge and a decrease in membrane resistance due to opening of kation channels. This response is inhibited by tropisetron and MDL 72222 and mimicked by 2-methyl-5-HT (Mawe *et al.*, 1986; Nemeth & Gullikson, 1989; see Galligan & North, 1989), suggesting that it is mediated by 5-HT_3 receptors. As described above, most benzamides are 5-HT_3 receptor antagonists. In electrophysiological experiments with myenteric neurons, cisapride, renzapride, zacopride and to a lesser extent metoclopramide have been demonstrated to inhibit the fast transient depolarization to 5-HT (Nemeth *et al.*, 1985; Nemeth & Gullikson, 1989; Wade *et al.*, 1991; Schemann, 1991). These results confirm the 5-HT_3 receptor antagonistic properties of the benzamides.

Interactions with 5-HT$_{1A}$ receptors and relation to motility effects

A second effect of 5-HT is hyperpolarization of a subset of neurons associated with a decrease in membrane resistance (opening of K^+-channels). This effect is now known to be mediated by 5-HT$_{1A}$ receptors (Wade *et al.*, 1991; Tack *et al.*, 1992; Pan & Galligan, 1994; see Galligan & North, 1989). Presynaptic suppression of fast excitatory postsynaptic potentials (EPSPs) recorded from S/type I-cells that are evoked by electrical stimulation of the ganglia or interganglionic fiber tracts (North *et al.*, 1980; Nemeth *et al.*, 1985; Tonini *et al.*, 1989) is also caused by 5-HT$_{1A}$ receptor stimulation. These fast EPSPs are mediated by acetylcholine acting on nicotinic (hexamethonium-sensitive) cholinoceptors. Thus, an inhibition of the fast EPSPs has been explained by a presynaptic inhibition of acetylcholine release (North *et al.*, 1980; Nemeth *et al.*, 1985).

High concentrations of cisapride (1-5 μM) suppressed or abolished the fast cholinergic EPSPs evoked by electrical stimulation of the interganglionic fiber tracts (Nemeth *et al.*, 1985). Furthermore, cisapride induced a hyperpolarization in some neurons at high concentrations (3-10 μM) (Tonini *et al.*, 1989). Galligan *et al.* (1988) found cisapride (1 μM) not to block 5-CT-induced presynaptic inhibition. Renzapride (1 μM) did not affect the hyperpolarization response or inhibition of the fast EPSPs induced by 5-HT (Tack *et al.*, 1992). Wade *et al.* (1994) showed that even 100 μM renzapride did not affect the 5-HT$_{1A}$ receptor-mediated response. Hence, renzapride and cisapride do not seem to interfere with 5-HT$_{1A}$ receptors up to a concentration of 1 μM, but at higher concentrations cisapride behaves as a (partial) 5-HT$_{1A}$ receptor agonist. These results have been confirmed in another electrophysiological study (Pan & Galligan, 1994). The low affinity of cisapride at 5-HT$_{1A}$ receptors in electrophysiological experiments, and also in radioligand binding studies (see Table 1.4), contrasts sharply with the potent antagonism (IC_{50} = 1.5 nM) of cisapride against the 5-HT-induced inhibition of field stimulation-induced cholinergic contractions of the guinea-pig ileum (Taniyama *et al.*, 1992), a response supposedly mediated by 5-HT$_{1A}$ receptors (Galligan, 1992). The electrophysiological and radioligand binding data are however in agreement with the observation of Pfeuffer-Friederich and Kilbinger (1984), who reported that 10 μM cisapride, but not lower concentrations, inhibited acetylcholine release from guinea-pig ileum strips, an effect which could be antagonized by methiothepin.

Could a 5-HT$_{1A}$ receptor mechanism account for the prokinetic effects of benzamides? Firstly, the concentration necessary for cisapride to have an action on 5-HT$_{1A}$ receptors (> 1 μM, see above) is not likely to be attained under therapeutical conditions. Renzapride seems to lack even low affinity for 5-HT$_{1A}$ recep-

tors (Wade *et al.*, 1994), while it does stimulate motility *in vivo* (see Table 1.2). Secondly, 5-HT_{1A} receptor agonism would lead to inhibition of cholinergic transmission, and selective 5-HT_{1A} receptor antagonists, like NAN-190 and spiperone, do not enhance field stimulation-induced cholinergic contractions in the guinea-pig ileum (Buchheit *et al.*, 1985b; Galligan, 1992). Therefore, a 5-HT_{1A} receptor mechanism is unlikely to account for the facilitation of cholinergic transmission, if it is assumed that the latter subserves the motor stimulating properties of the benzamides. Moreover, methiothepin (5-HT_1/5-HT_2 receptor antagonist) and spiperone did not affect gastric emptying in the guinea-pig (Buchheit *et al.*, 1985a). Therefore, the prokinetic action of the benzamides is not considered to involve a 5-HT_{1A} receptor mechanism.

Interactions with 5-HT_{1P} receptors and relation to motility effects

A third direct effect of 5-HT, found exclusively in AH/type II neurons, is a slow long lasting depolarization associated with an increase of membrane resistance (Mawe *et al.*, 1986; Nemeth *et al.*, 1985). These effects are mimicked by 5-hydroxyindalpine (5-OHIP), 6-hydroxyindalpine (6-OHIP) and 2-Me-5-HT, and inhibited by methysergide. The dipeptide of 5-hydroxytryptophan, 5-hydroxytryptophyl-5-hydroxytryptophanamide (5-HTP-DP), is a specific antagonist (Mawe *et al.*, 1986; Takaki *et al.*, 1985). This receptor was called 5-HT_{1P} ("P" of "Peripheral"; Mawe *et al.*, 1986) as it does not resemble any of the currently known 5-HT receptors with respect to its pharmacological profile (it does *not* belong to the 5-HT_1 receptor family). Slow EPSPs in a subset of neurons after stimulation of interganglionic nerve tracts are thought to be due to release of 5-HT acting on 5-HT_{1P} receptors (see Galligan & North, 1989; Gershon & Wade, 1993).

Cisapride (0.1-5 μM) reversibly suppressed the prolonged excitatory responses to 5-HT in AH/type II neurons, as well as the slow EPSPs due to focal electrical stimulation of the nerve fibers, suggesting it is an antagonist of 5-HT_{1P} receptors (Nemeth *et al.*, 1985). Renzapride (0.5-1 μM) and R-zacopride mimicked this action of cisapride (Mawe *et al.*, 1989; Wade *et al.*, 1991) and can thus also be considered 5-HT_{1P} receptor antagonists. In contrast, S-zacopride was found to mimick the 5-HT-induced 5-HT_{1P} receptor-mediated slow depolarization (EC_{50} = 1 μM). This effect to S-zacopride was sensitive to 5-HT desensitization and to inhibition by renzapride and 5-HTP-DP, suggesting S-zacopride is a 5-HT_{1P} receptor agonist (Wade *et al.*, 1991). Slow depolarization responses due to 5-HT_{1P} receptor stimulation by 5-HT of benzamides were not inhibited by tropisetron up to 10 μM, a concentration which blocks 5-HT_3 as well as 5-HT_4 receptors (Wade &

Gershon, 1991). Hence, whereas S-zacopride is a 5-HT_{1P} receptor agonist, R-zacopride, cisapride and renzapride act as antagonists on 5-HT_{1P} receptors.

Is there a relation between the effects of the benzamides on 5-HT_{1P} receptors and gastrointestinal prokinetic activity *in vivo*? Mawe *et al.* (1989) found renzapride, a 5-HT_3/5-HT_{1P} receptor antagonist and a 5-HT_4 receptor agonist, to enhance the rate of gastric emptying, but not the transit through the small intestine of a ^{51}Cr-labeled meal of conscious mice. In contrast, tropisetron in a 5-HT_3 receptor blocking dose did not accelerate gastric emptying, but the 5-HT_{1P} receptor antagonist 5-HTP-DP did. From these results the authors concluded that antagonism of 5-HT_{1P} receptors caused the acceleration of gastric emptying (Mawe *et al.*, 1989). However, 5-HTP-DP (a dipeptide) might be metabolized to 5-hydroxytryptophan or other products, causing acceleration of gastric emptying through a non-5 HT_{1P} mechanism (Banner *et al.*, 1993). Moreover, at the time, 5-HT_4 receptors were not yet known. Furthermore, electrophysiology of myenteric neurons and thus of 5-HT_{1P} has always been done in guinea-pig ileum and stomach, and is not necessarily relevant for gastrointestinal motility in the mouse. Thus, these results do not convince that 5-HT_{1P} receptors are important in the prokinetic action of benzamides.

Propulsion of a solid pellet in isolated guinea-pig distal colon was inhibited by 5-HTP-DP (IC_{50} = 30 μM), renzapride and tropisetron (IC_{50} = 5 μM), but not by R-zacopride and granisetron. From these observations the authors concluded that 5-HT_{1P} receptor and 5-HT_4 receptors participate in mediating the peristaltic reflex *in vitro* (Wade & Gershon, 1991). However, the extremely high concentration of 5-HTP-DP, necessary to inhibit the reflex raises questions as to its specificity for 5-HT_{1P} receptors. Furthermore, like renzapride, R-zacopride was reported by the same authors to be a 5-HT_{1P} receptor antagonist (Wade *et al.*, 1991), but in contrast to the 5 HT_{1P} receptor antagonist 5-HTP-DP, it did not affect propulsion. Thus, the arguments for involvement of a 5-HT_{1P} receptor mechanism are weak. Buchheit & Buhl (1993) tested the effects of the 5-HT_{1P} receptor agonist 6-OHIP (Mawe *et al.*, 1986) on the peristaltic reflex of the guinea-pig ileum, using the Trendelenburg technique. However, 6-OHIP had no effect up to 10 μM (Buchheit & Buhl, 1993), suggesting a lack of importance of 5-HT_{1P} receptors in this response.

Interactions with 5-HT_4 receptors and post-receptor mechanisms

Although direct electrophysiological actions to 5-HT on enteric neurons such as depolarization, hyperpolarization and change in membrane conductance have been found to be due to 5-HT_3, 5-HT_{1A} and 5-HT_{1P} receptor stimulation, no such

direct effects due to 5-HT_4 receptor stimulation have been observed to date. Tonini *et al.* (1989) showed, that cisapride (10 nM - 1 μM) can enhance the fast cholinergic EPSPs after fiber tract stimulation recorded from S neurons. Pan and Galligan (1994) found that not only cisapride, but also renzapride, 5-MeOT and the 5-HT_4 receptor agonist BIMU 8 amplified the electrical stimulation-evoked cholinergic EPSPs. Furthermore, tropisetron (1 μM) but not ondansetron (1 μM) could inhibit the potentiating effects of these compounds (Tonini *et al.*, 1989; Pan and Galligan, 1994). These results strongly suggest that 5-HT_4 receptors are present on the enteric nerves, whose stimulation causes an increase in acetylcholine release. This observation parallels the observation made in functional models of the guinea-pig gut, as described before.

5-HT_4 receptors in mouse colliculi neurons, guinea-pig hippocampal membranes, rat oesophagus and in human heart and frontal cortex have been shown to be positively linked to adenylate cyclase (Dumuis *et al.*, 1988; Bockaert *et al.*, 1990; Kaumann *et al.*, 1990; Ford *et al.*, 1992; Monferini *et al.*, 1993). Stimulation of enteric 5-HT_4 receptors in association with cholinergic facilitation has been reported from preliminary experiments to involve cAMP and K^+ channels (Yau & Youther, 1993). In mouse colliculi neurons, stimulation of 5-HT_4 receptors inhibited K^+ current *via* activation of a cyclic AMP-dependent protein kinase (Fagni *et al.*, 1992). Extrapolating, such an action on K^+ current could cause a slow membrane depolarization as shown after 5-HT_4 receptor stimulation in rat hippocampal pyrimidal CA1 neurons (Andrade & Chaput, 1991). An elevation of cAMP levels in the cell has been shown to mimick slow synaptic excitation and an enhanced excitability in myenteric neurons of the guinea-pig (Nemeth *et al.*, 1986; Palmer *et al.*, 1986). However, in most electrophysiological studies on myenteric neurons, cisapride, renzapride and BIMU 8 did not have an effect on the membrane potential up to 1 μM (Tonini *et al.*, 1989; Mawe *et al.*, 1989; Pan & Galligan, 1994). Metoclopramide, cisapride and renzapride slightly depolarized (< 10 mV) some cells in other studies but it was not verified whether this was due to 5-HT receptor stimulation (Nemeth *et al.*, 1985; Nemeth & Gullikson, 1989). R-Zacopride never caused more than 2 mV depolarization in AH/type II neurons in the study by Wade *et al.* (1991). Thus, it has been postulated, but not proven, that at least the majority of the 5-HT_4 receptors are not located on the soma, where the membrane potential is measured, but rather on the processes or nerve terminals (see: Tonini *et al.*, 1991).

Smooth muscle effects

It has been reported that part of the contraction to cisapride on the isolated guinea-pig colon is caused by a direct action on the smooth muscle without involvement of 5-HT receptors (Nakayama *et al.*, 1985; Schuurkes *et al.*, 1985b; Briejer *et al.*, 1993b). Den Hertog & Van den Akker (1986) studied the effects of cisapride on smooth muscle preparations of the taenia coli, both mechanically and with the sucrose-gap method. Cisapride caused depolarization, enhancement of spike activity, increase in muscle tone and potentiation of the contraction evoked by transmural electrical stimulation. These effects were only partially inhibited by atropine and TTX. Inhibitory junction potentials represented by hyperpolarization and the rebound response were also enhanced. It was concluded from these and other observations, that cisapride inhibits calcium extrusion from the smooth muscle cells by a direct action, leading to depolarization and thus to enhanced excitability (Den Hertog & Van den Akker, 1986). Using glass intracellular microelectrodes impaled on guinea-pig stomach circular smooth muscle, cisapride was found to cause a depolarization of the smooth muscle membrane (Ohno *et al.*, 1993). These electrophysiological actions on the smooth muscle of cisapride could well parallel the mechanical effects described above (Nakayama *et al.*, 1985; Schuurkes *et al.*, 1985b; Briejer *et al.*, 1993b). In contrast to the above described observations with cisapride on guinea-pig intestinal smooth muscle, renzapride did not affect the membrane potential of circular smooth muscle cells of the guinea-pig ileum (King & Sanger, 1992).

Hasler *et al.* (1991) observed that cisapride contracts isolated smooth muscle cells from the guinea-pig stomach. From their results they concluded that these effects could be mediated by glandular M_2 cholinoceptors (currently called M_3 cholinoceptors) linked to the calcium-phosphoinositide pathway. However, the relative ineffectiveness of atropine against the cisapride-induced contraction of the isolated smooth muscle cells undermines these conclusions. It is unclear how these results relate to the findings, as described above.

Thus it is clear that cisapride has direct stimulating effects on gastrointestinal smooth muscle tissue. It is however unclear how these effects are induced, and whether they are relevant for its prokinetic effects. The sensitization to direct smooth muscle contracting agonists after metoclopramide and cisapride, as previously described, might be related to a partial depolarization of the smooth muscle by these benzamides.

Radioligand binding studies

An overview of binding affinities of several benzamides for binding sites of 5-HT and other endogenous neurotransmitters is given in Table 1.4. The recent development of the selective and highly potent 5-HT_4 receptor antagonists GR 113808 and SB 207710 has allowed the development of a 5-HT_4 receptor radioligand binding assay (Brown *et al.*, 1993a; Grossman *et al.*, 1993). The benzamides displaced [^{3}H]-GR 113808 and [^{125}I]-SB 207710 binding on 5-HT_4 sites in guinea-pig and piglet brain with affinities varying from pK_i = 5.5 to 7.7. Also in this kind of studies, benzamides such as cisapride, SC 53116 and renzapride are more potent than metoclopramide. Most benzamides also bind with moderate to high potency at 5-HT_3 receptor sites (see Tables 1.3 and 1.4). Cisapride, but not the other benzamides listed in Table 1.4, has repeatedly been reported to bind with high affinity at 5-HT_{2A} receptor sites, and also in functional studies it behaved as a potent 5-HT_{2A} receptor antagonist (Moriarty *et al.*, 1987). As other benzamides, such as zacopride, renzapride and SC-53116 lack affinity for 5-HT_{2A} receptors, 5-HT_{2A} receptor antagonism cannot be the common property to explain the prokinetic effects of the benzamides. Similar observations can be made with respect to affinity for 5-HT_{1A} binding sites. Also in electrophysiological experiments, cisapride had detectable (micromolar) affinity for these sites whereas renzapride had no affinity (see paragraph on 5-HT_{1A} receptors). For adrenergic binding sites the benzamides lack affinity, except for cisapride, which binds to α_1-adrenoceptors. Clebopride binds potently at dopamine D_2 receptors, while cisapride and metoclopramide have only moderate affinity. Binding at other sites (histamine, acetylcholine and opioid binding sites) is low as far as data are available (see Table 1.4).

In rabbit ileum, [^{3}H]-5-HT binding was not affected by 5-MeOT, but it was inhibited by the 5-HT_{1P} receptor antagonist 5-HTP-DP (Takaki *et al.*, 1985). In the similar preparation, also the 5-HT_{1P} receptor agonist [^{3}H]-5-OHIP bound with high affinity, and this binding could be displaced by 5-HT, 2-Me-5-HT, and 5-HTP-DP, but not by 5-MeOT or tropisetron (K_i > 500 nM) (Branchek *et al.*, 1988). In guinea-pig small intestinal preparations, similar results have been obtained (Mawe *et al.*, 1989). Furthermore, it has been shown that renzapride (up to 50 µM), and R- and S-zacopride do not affect [^{3}H]-5-HT binding (Mawe *et al.*, 1989; Wade *et al.*, 1991). However, radioautography studies showed that [^{3}H]-zacopride binds at specific sites in guinea-pig, rat and rabbit intestine. An effective displacer of this [^{3}H]-zacopride binding was renzapride, while tropisetron and 5-HTP-DP were partially effective. In the presence of tropisetron, R- and S-zacopride and

renzapride still caused inhibition of [^{3}H]-zacopride binding, but 5-HTP-DP did not (Wade *et al.*, 1991). These results suggest that in the gut, with respect to the compounds used, at least two binding sites exist. Firstly, binding sites are present which recognize 5-HT, 5-HTP-DP and 5-OHIP, but not tropisetron and 5-MeOT, suggesting that these sites correspond to 5-HT_{1P} rather than to 5-HT_4 receptors. The benzamides do not bind on these 5-HT_{1P} sites. Secondly, binding sites are present which selectively recognize benzamides such as renzapride and the stereoisomers of zacopride. These sites do not recognize 5-HT or tropisetron, and therefore, these sites are specific benzamide sites, which after occupation prevent binding at 5-HT_{1P} sites.

In mouse colliculi neurons, Bockaert and coworkers (1991) showed that [^{3}H]-renzapride binding could be inhibited by the benzamides R 76186, metoclopramide and zacopride, confirming that also in these cells benzamide binding sites are present. As in the gut, 5-HT and 5-MeOT could not displace [^{3}H]-renzapride binding, though the benzimidazolone 5-HT_4 receptor agonist BIMU 8 displaced [^{3}H]-renzapride binding partially, suggesting that renzapride binds on at least two binding sites. In contrast to the findings described above with [^{3}H]-zacopride binding in the gut, in the mouse colliculi neurons [^{3}H]-renzapride binding could be inhibited by tropisetron but not by 5-HT_3 receptor antagonists (Bockaert *et al.*, 1991). Hence, in mouse colliculi neurons benzamides and 5-HT bind to different binding sites, though functional effects (elevation of cAMP levels) can both be inhibited by micromolar concentrations of tropisetron. These two sites could both be subsites of the 5-HT_4 receptor, and occupation of either site might activate the second messenger system. The 5-HT_4 receptor antagonists tropisetron, DAU 6285 and SDZ 205557 have been shown to be competitive antagonists against 5-HT-induced responses in several tissues. However, against benzamide-induced responses in some reports non-competitive inhibition has been described (Elswood *et al.*, 1991; Buchheit *et al.*, 1992; Briejer *et al.*, 1993b). These observations could be explained with 5-HT and benzamides not binding on the same site of the 5-HT_4 receptor molecule.

Table 1.4 Receptor binding affinities (pK_i) as obtained from radioligand displacement studies on 5-hydroxytryptamine (5-HT) binding sites.

	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_1	5-HT_{2A}	5-HT_{2C}	5-HT_3	5-HT_4	ref
cisapride					8.3		6.8	7.5	1
							6.9	7.5	2
				< 5	8.2		6.9		5
								7.2/7.5	6
					8.6	5.6			7
				5.3	8.1		5.8		9
	6.1	< 5*	< 5*		8.6	5.8	6.4		10
								7.2	11
metoclopramide							6.4	6.0	3
							6.9	6.0	2
								6.2/6.3	6
				< 5	6.0		6.6		9
								5.5	11
renzapride							8.6	6.9	2
				< 5	< 5		8.3		5
	< 5	< 5	< 5		< 5	< 5	8.4		4
								6.9/7.0	6
								6.2	11
R,S-zacopride							9.2	6.1	3
							9.5	6.4	2
	< 5	< 5	< 5		< 5	5.9	9.3		4
								6.7/6.8	6
R-zacopride							8.8	5.7	2
				< 5	6.3		8.1		5
								6.0/6.3	6
S-zacopride							10.0	6.5	2
				< 5	< 5		9.6		5
								6.6/6.7	6
SC-53116				< 5	< 5		6.8		5
							7.3	7.7	2
clebopride					6.7				8

Table 1.4 (*Continued*) Receptor binding affinities (pK_i) as obtained from radioligand displacement studies on respectively adrenergic, dopamine, histamine, acetylcholine and opioid binding sites.

	α_1	α_2	β	D_1	D_2	H_1	ACh	μ	ref
cisapride	8.0				6.7				1
	7.5	5.3	< 5	5.8	6.6				5
					6.6				9
	7.4	< 5*	< 5*	< 5*	7.0	5.9	< 5*	5.9	10
metoclopramide					6.8				9
renzapride	< 5	< 5	< 5	< 5	< 5				5
	< 5	< 5	< 5			< 5	< 5	< 5	4
R,S-zacopride	< 5	< 5	< 5			< 5	< 5	< 5	4
R-zacopride	< 5	< 5	< 5	< 5	< 5				5
S-zacopride	< 5	< 5	< 5	< 5	< 5				5
SC-53116	< 5	< 5	< 5	< 5	< 5				5
clebopride	5.8	6.1		< 5	8.5	< 5	< 5	< 5	8

*pIC_{50} value instead of pK_i value. References: (1) Rizzi *et al.*, 1994; (2) Schiavi *et al.*, 1994; (3) Langlois *et al.*, 1994; (4) van Wijngaarden et al., 1990; (5) Flynn *et al.*, 1992; (6) Grossman *et al.*, 1993; (7) Leysen, 1990; (8) Takeda *et al.*, 1991; (9) Linnik *et al.*, 1991; (10) Heylen *et al.*, 1992; (11) Brown *et al.*, 1993a.

In rat brain, and later also in human brain, but also in the gastrointestinal tract (and many other tissues), high affinity R-zacopride binding sites have been found which do not recognize S-zacopride, and which do not represent 5-HT_3 binding sites (Kidd *et al.*, 1992; 1993). [^{3}H]-R-zacopride binding could not be inhibited by 5-HT, dopamine, histamine, noradrenaline, and many other (neurotransmitter) compounds. Hence, this 'R-site' does not seem to be related to a known neurotransmitter receptor. Only prazosin and mianserin showed moderate affinity for the 'R-site' (Kidd *et al.*, 1992; 1993). Unfortunalely, no data with respect to displacement of [^{3}H]-R-zacopride binding by other prokinetic benzamides are available. It is therefore difficult to evaluate the pharmacological and therapeutical relevance of the 'R-site' binding.

Conclusions and unsolved issues

Though the first generation benzamides, i.e. metoclopramide, bromopride and clebopride, are antagonists of peripheral dopamine D_2 receptors, the second generation benzamides (i.e. cisapride, renzapride) have only moderate or no affinity for these sites. Antagonism at D_2 receptors is not likely to explain in

general the gastrointestinal motility-stimulating action of benzamides. Most benzamides share moderate to high affinity for 5-HT_3 receptors, acting as antagonists. Also this property is very unlikely to account for their prokinetic effects, although it might contribute in some species, e.g. the rat. Benzamides have been shown to facilitate cholinergic transmission by causing an increase in acetylcholine release in the guinea-pig enteric nervous system, though a similar action could not be found in the rat. In this species, 5-HT_4 receptors on the intestinal smooth muscle cells mediate relaxation in contrast to the situation in the guinea-pig where 5-HT_4 receptors on the enteric nerves mediate (cholinergic) contraction. As benzamides are moderate affinity agonists at these receptors, facilitation of cholinergic transmission has been shown to be due to agonism at the 5-HT_4 receptors in the guinea-pig. Despite the above mentioned species differences between the rat and guinea-pig, benzamides have been shown to stimulate motility in guinea-pigs and also seem to do so in the rat, although in the latter species data are scarce. Therefore it is encouraged that more *in vivo* studies in the rat are conducted.

In the dog, and also in man, benzamides seem to enhance gastric emptying and gastroduodenal motility through stimulation of neuronal 5-HT_4 receptors on cholinergic nerves. It has to date not yet been established whether the increase in lower oesophageal sphincter pressure after cisapride is due to neuronal 5-HT_4 receptor stimulation. Although for the dog no data on the effects of benzamides on lower gut motility are available, in man cisapride stimulates colonic motility and is moderately effective in constipation. However, smooth muscle but no neuronal 5-HT_4 receptors have been found in the isolated human colon, causing inhibition of spontaneous movements and relaxation through an acetylcholine-independent mechanism. Similar *in vitro* results have been obtained with other benzamides as well. A mechanism additional to 5-HT_4 receptor agonism is necessary to explain the observations on lower gut motility in patients.

Facilitation of non-cholinergic transmission, of which only few data exist, would be a direction worthwhile to explore. Contractions to exogenous neurotransmitter substances, especially to acetylcholine and histamine, have in several tissues been found to be amplified by benzamides, although very high (non-therapeutic) concentrations were often necessary. Nevertheless, more investigation into these phenomena may prove worthwhile. Benzamides interfere with the putative 5-HT_{1P} receptors, but a role for these receptors in gastrointestinal motility has to be convincingly demonstrated yet. Several lines of evidence suggest that the 5-HT_4 receptor has separate binding sites for 5-HT and related indoles on one hand and benzamides on the other hand. More investiga-

tion into the exact interrelations between these sites and 5-HT_{1P} receptors would be interesting. In relation to this, the exact location and alterations in cellular function after stimulation of these receptors should be determined. Lastly, direct effects on the smooth muscle need clarification.

References

Akkermans LMA, Vos A, Hoekstra A, Roelofs JMM, Horowitz M (1988) Effect of ICS 205-930 (a specific 5-HT_3 receptor antagonist) on gastric emptying of a solid meal in normal subjects. *Gut* 29: 1249-1252.

Altaparmakov I (1985) Non-cholinergic stimulation of contractile activity of human colon by a novel gastrointestinal prokinetic cisapride. *Naunyn-Schmiedeberg's Arch Pharmacol* 330: R77.

Andrade R, Chaput Y (1991) 5-Hydroxytryptamine$_4$-like receptors mediate the slow excitatory response to serotonin in the rat hippocampus. *J Pharmacol Exp Ther* 257: 930-937.

Andrews PL, Rapeport WG, Sanger GJ (1988) Neuropharmacology of emesis induced by anti-cancer therapy. *Trends Pharmacol Sci* 9: 334-341.

Banner SE, Smith MI, Sanger GJ (1993) 5-HT receptors and 5-hydroxytryptophan-evoked defecation in mice. *Br J Pharmacol* 110 suppl: 135P.

Baxter GS, Craig DA, Clarke DE (1991) 5-Hydroxytryptamine$_4$ receptors mediate relaxaton of the rat oesophageal tunica muscularis mucosa. *Naunyn-Schmiedeberg's Arch Pharmacol* 343: 439-446.

Beani L, Bianchi C, Crema C (1970) Effects of metoclopramide on isolated guinea-pig colon. 1) Peripheral sensitization to acetylcholine. *Eur J Pharmacol* 12: 320-331.

Bianchi C, Beani L, Crema C (1970) Effects of metoclopramide on isolated guinea-pig colon. 2) Interference with ganglionic stimulant drugs. *Eur J Pharmacol* 12: 332-341.

Bingham S, Andrews PLR (1987) Cisapride enhances nonadrenergic non-cholinergic motility in the gastric antrum of the anaethetized ferret. *Gastroenterology* 92: 1317.

Bingham S, Andrews PLR (1992) Gastrointestinal motility effects of cisapride mediated by nonadrenergic, noncholinergic nerves. In: *Gastrointestinal dysmotility: focus on cisapride* (Heading RC, Wood JD Eds.) Raven Press New York, pp. 93-106.

Bingham S, King BF, Rushant B, Smith MI, Sanger GJ (1993) The 5-HT_4 receptor antagonist SB 204070 inhibits the contractile response to 5-HT in the dog heidenhain pouch. *Br J Pharmacol* 110 suppl.: 16P.

Birtley RDN, Baines MW (1973) The effects of metoclopramide on some isolated intestinal preparations. *Postgrad Med J* (July suppl.): 13-18.

Bockaert J, Sebben M, Dumuis A (1990) Pharmacological characterization of 5-hydroxytryptamine$_4$ (5-HT_4) receptors positively coupled to adenylate cyclase in adult guinea pig hippocampal membranes: effect of substituted benzamide derivatives. *Mol Pharmacol* 37: 408-411.

Bockaert J, Fagni L., Sebben M, Dumuis A (1991) Pharmacological characterization of brain 5-HT_4 receptors: relationship between the effects of indole, benzamide and azabicycloalkyl-benzimidazolone derivatives. In: *Serotonin: molecular biology, receptors and functional effects* (Fozard JR, Saxena PR Eds.) Birkhäuser Verlag Basel, Switzerland, pp 220-231.

Bornstein JC, Furness JB, Kunze WAA (1994) Electrophysiological characterization of myenteric neurons: how do clasification schemes relate? *J Auton Nerv Syst* 48: 1-15.

Bou J, Fernández AG, Llupiá J, Puig J, Berga P (1988) Cinitapride: a new benzamide with a procholinergic, intestinal motility modulatory profile of activity. *Rev Farmacol Clin Exp* 5: 254.

Boyd H, Burnstock G, Campbell G, Jowett A, O'Shea J, Wood M (1963) The cholinergic blocking action of adrenergic blocking agents in pharmacological analysis of autonomic innervation. *Br J Pharmacol Chemother* 20: 418-435.

Bradley PB, Engel G, Feniuk W, Fozard JR, Humphrey PPA, Middlemiss DN, Mylecharane EJ, Richardson BP, Saxena PR (1986) Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology* 25: 563-576.

Branchek TA, Mawe GM, Gershon MD (1988) Characterization and localization of a peripheral neural 5-hydroxytryptamine receptor subtype (5-HT_{1P}) with aselective agonist, ^{3}H-5-hydroxytryptamine. *J Neurosci* 8: 2582-2595.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1993a) Pharmacological characterization of 5-hydroxytryptamine receptor types in the guinea-pig proximal colon. *J Gastroint Motil* 5: 239-246.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1993b) Cisapride and a structural analogue, R 76186, are 5-hydroxytryptamine$_4$ (5-HT_4) receptor agonits on the guinea-pig colon ascendens. *Naunyn-Schmiedeberg's Arch Pharmacol* 347: 464-470.

Briejer MR, Veen GJ, Akkermans LMA, Schuurkes JAJ (1994) Cisapride facilitates inhibitory purinergic transmission in the guinea-pig colon. *Gastroenterology* 106: A471.

Brittain RT, Butler A, Coates IH, Forture DH, Hagan R, Hill JM, Humber DC, Humphrey PPA, Ireland SJ, Jack D, Jordan CC, Oxford A, Straughan DW, Tyers MB (1987) GR 38032F, a novel selective 5-HT_3 receptor antagonist. *Br J Pharmacol* 90: 87P

Brogden RN, Carmine AA, Heel RC, Speight TM, Avery GS (1982) Domperidone. A review of its pharmacological activity, pharmacokinetics and therapeutic efficacy in the symptomatic treatment of chronic dydpepsia and as an antiemetic. *Drugs* 24: 360-400.

Brown AM, Young TJ, Patch TL, Cheung CW, Kaumann AJ, Gaster L, King FD (1993a) [^{125}I]-SB 207710, a potent, selective radioligand for 5-HT_4 receptors. *Br J Pharmacol* 110 suppl.: 10P.

Brown NJ, Horton A, Rumsey RDE, Read NW (1993b) Granisetron and ondansetron: effects on the ileal brake mechanism in the rat. *J Pharm Pharmacol* 45: 521-524.

Buchheit KH, Bertholet A (1992) 5-HT effects on isolated strips from guinea pig stomach. *Naunyn-Schmiedeberg's Arch Pharmacol* 345 suppl.: R111.

Buchheit KH, Buhl T (1991) Prokinetic benzamides stimulate peristaltic activity in the isolated guinea pig ileum by activation of 5-HT_4 receptors. *Eur J Pharmacol* 205: 203-208.

Buchheit KH, Buhl T (1993) 5-HT receptor subtypes involved in the stimulatory effect of 5-HT on the peristaltic reflex *in vitro*. *J. Gastroint. Mot.* 5: 49-55.

Buchheit KH, Costall B, Engel G, Gunning SJ, Naylor RJ, Richardson BP (1985a) 5-Hydroxytryptamine receptor antagonism by metoclopramide and ICS 205-930 in the guinea-pig leads to enhancement of contractions of stomach muscle strips induced by electrical field stimulation and facilitation of gastric emptying *in vivo*. *J Pharm Pharmacol* 37: 664-667.

Buchheit KH, Engel G, Mutschler E, Richardson B (1985b) Study of the contractile effect of 5-hydroxytryptamine (5-HT) in the isolated longitudinal muscle strip from the guinea-pig ileum. *Naunyn-Schmiedeberg's Arch Pharmacol* 329: 36-41.

Buchheit KH, Gamse R, Pfannkuche HJ (1992) SDZ 205-557, a selective, surmountable antagonist for 5-HT_4 receptors in the isolated guinea pig ileum. *Naunyn-Schmiedeberg's Arch Pharmacol* 345: 387-393.

Buckley NJ, Burnstock G (1986) Autoradiographic localization of peripheral M_1 muscarinic receptors using [^{3}H]-pirenzepine. *Brain Res* 375: 83-91.

Burleigh DE (1977) Evidence for more than one type of 5-hydroxytryptamine receptor in the human colon. *J Pharm Pharmacol* 29: 538-541.

Burleigh DE, Trout SJ (1985) Evidence against an acetylcholine releasing action of cisapride in the human colon. *Br J Clin Pharmacol* 20: 475-478.

Burnstock G (1990) Co-transmission. *Arch Int Pharmacodyn Ther* 304: 7-33.

Butler A, Elswood CJ, Burridge J, Ireland SJ, Bunce KT, Kilpatrick GJ, Tyers MB (1990) The pharmacological characterization of 5-HT_3 receptors in three isolated preparations derived from guinea-pig tissues. *Br J Pharmacol* 101: 591-598.

Burke TA, Sanger GJ (1988) Regionally selective cholinergic stimulation by BRL 24924 in the human isolated gut. *Br J Clin Pharmacol* 26: 261-265.

Bury RW, Mashford ML (1976) The effects of metoclopramide in modifying the response of isolated guinea-pig ileum to various agonists. *J Pharmacol Exp Ther* 197: 641-646.

Clayton NM, Gale JD (1994) Prokinetic benzamides do not stimulates small intestinal transit in the conscious rat. *Neurogastroenterol Motil* 6: 149P.

Cohen ML, Bloomquist W, Gidda JS, Lacefield W (1989) Comparison of the 5-HT_3 receptor natagonist properties ICS 205-930, GR 38032F and zacopride. *J Pharmacol Exp Ther* 248: 197-201.

Cohen ML, Bloomquist W, Gidda JS, Lacefield W (1990) LY277359 maleate: a potent and selective 5-HT_3 receptor antagonist without gastroprokinetic activity. *J Pharmacol Exp Ther* 254: 350-355.

Costall B, Naylor RJ, Tan CCW (1983) Field stimulation-induced responses of circular smooth muscle from guinea-pig stomach. Characterisation of the receptor mechanisms involved. *Naunyn-Schmiedeberg's Arch Pharmacol* 323: 155-161.

Costall B, Naylor RJ, Tan CCW (1984) Neuronally mediated contraction responses of guinea-pig stomach smooth muscle preparations: modification by benzamide derivatives does not reflect a dopamine antagonist action. *Eur J Pharmacol* 102: 79-89.

Costall B, Kelly ME, Naylor RJ, Tan CCW, Tattersail FD (1986) 5-Hydroxytryptamine M receptor antagonism in the hypothalamus facilitates gastric emptying in the guinea-pig. *Neuropharmacology* 25: 1293-1296.

Costall B, Gunning SJ, Naylor RJ, Tyers MB (1987) The effect of GR 38032F, a novel 5-HT_3 receptor antagonist on gastric emptying in the guinea-pig. *Br J Pharmacol* 91: 263-264.

Cox B, Ennis C (1980) Mechanism of action of dopamine on the guinea-pig isolated gastroduodenal junction.*Br J Pharmacol* 68: 180P.

Craig DA, Clarke DE (1990) Pharmacological characterization of a neuronal receptor for 5-hydroxytryptamine in guinea pig ileum with properties similar to the 5-hydroxytryptamine$_4$ receptor. *J Pharmacol Exp Ther* 252: 1378-1386.

Craig DA, Clarke DE (1991) Peristalsis evoked by 5-HT and renzapride: evidence for putative 5-HT_4 receptor activation. *Br J Pharmacol* 102: 563-564.

Craig DA, Eglen RM, Walsh LKM, Perkins LA, Whiting RL, Clarke DE (1990) 5-Methoxytryptamine and 2-methyl-5-hydroxytryptamine-induced desensitization as a discriminative tool for the 5-HT_3 and putative 5-HT_4 receptors in the guinea pig ileum. *Naunyn-Schmiedeberg's Arch Pharmacol* 342: 9-16.

Day M, Vane JR (1963) An analysis of the direct and indirect actions of drugs on the isolated guinea-pig ileum. *Br J Pharmacol* Chemother 20: 150-170.

Den Hertog A, Van den Akker J (1986) The effect of cisapride on smooth muscle cells of guinea-pig taenia caeci. *Eur J Pharmacol* 126: 31-35.

De Ridder WJE, Schuurkes JAJ (1993) Cisapride and 5-hydroxytryptamine enhance motility in the canine antrum *via* separate pathways, not involving 5-hydroxytryptamine$_{1,2,3,4}$ receptors. *J Pharmacol Exp Ther* 264: 79-88.

Dumuis A, Bouhelal R, Sebben M, Cory R, Bockaert J (1988) A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol Pharmacol* 34: 880-887.

Dumuis A, Sebben M, Bockaert J (1989a) BRL 24924: A potent agonsit at a non-classical 5-HT receptor positively coupled to adenylate cyclase in colliculi neurons. *Eur J Pharmacol* 162: 381-384.

Dumuis A, Sebben M, Bockaert J (1989b) The gastrointestinal prokinetic benzamide derivatives are agonists at the non-classical 5-HT receptor (5-HT$_4$) positively coupled to edenylate cyclase in neurons. *Naunyn-Schmiedeberg's Arch Pharmacol* 340: 403-410.

Eglen RM, Swank SR, Walsh LKM, Whiting RL (1990) Characterization of 5-HT$_3$ and "atypical" 5-HT receptors mediating guinea-pig ileal contractions *in vitro*. *Br J Pharmacol* 101: 513-520.

Eisner M (1968) Gastrointestinal effects of metoclopramide in man. *In vitro* experiments with human smooth muscle preparations. *Br Med J* 4: 679-680.

Ellis ES, Wardle KA, Baxter GS, Sanger GJ (1994) 5-HT$_4$ receptor mediated contractions in guinea-pig distal colon circular muscle. *Br J Pharmacol* 112 suppl: 103P

Elswood CJ, Bunce KT, Humphrey PPA (1991) Identification of putative 5-HT$_4$ receptors in guinea-pig ascending colon. *Eur J Pharmacol* 196: 149-155.

Engel G, Hoyer D, Kalkman HO, Wick MB (1984) Identification of 5-HT$_2$ receptors on longitudinal muscle of the guinea pig ileum. *J Recep Res* 4: 113-126.

Engel G, Hoyer D, Kalkman HO, Wick MB (1985) Pharmacological similarity between the 5-HT D-receptor on the guinea-pig ileum and the 5-HT$_2$ binding site. *Br J Pharmacol* 84: 106P.

Ennis C, Schnieden H, Cox B (1978) Effects of the antiemetic drug domperidone on guinea-pig isolated gastrointestinal tissue. *J Pharm Pharmacol* 30: 34P.

Ennis C, Cox B (1980) The dopamine receptor antagonist domperidone is also a competitive antagonist at α_1-adrenoceptors. *J Pharm Pharmacol* 32: 434-435.

Fagni L, Dunuis A, Sebben M, Bockaert J (1992) The 5-HT$_4$ receptor subtype inhibits K^+ current in colliculi neurons *via* activation of a cyclic AMP-dependent protein kinase. *Br J Pharmacol* 105: 973-979.

Flynn DL, Zabrowski DL, Becker DP, Nosal R, Villamil CI, Gullikson GW, Moummi C, Yang DC (1992) SC-53116: the first selective agonist at the newly identified serotonin 5-HT$_4$ receptor subtype. *J Med Chem* 35: 1486-1489.

Fontaine J, Reuse J (1972) Stimulation transmural de l'ileon de cobaye: action du métoclopramide et du sulpiride. *Arch Int Pharmacodyn Ther* 197: 396-398.

Fontaine J, Reuse J (1973) Pharmacological analysis of the effects of metoclopramide on the guinea-pig ileum *in vitro*. *Arch Int Pharmacodyn Ther* 204: 293-305.

Fontaine J, Reuse J (1979) Pharmacological analysis of the effects of metoclopramide on the transmurally stimulated guinea-pig ileum. *Arch Int Pharmacodyn Ther* 242: 149-158.

Ford APDW, Baxter GS, Eglen RM, Clarke DE (1992) 5-Hydroxytryptamine stimulates cyclic AMP formation in the tunica muscularis mucosae of the rat oesophagus *via* 5-HT$_4$ receptors. *Eur J Pharmacol* 211: 117-120.

Forster ER, Dockray GJ (1990) The effect of ondansetron on gastric emptying in the conscious rat. *Eur J Pharmacol* 191: 235-238.

Fox AJ, Morton IKM (1991) An examination of the proposed functional interaction of substance P and 5-HT in the guinea-pig ileum. *Ann NY Acad Sci* 632: 385-388.

Fozard JR (1984) MDL 72222, a potent and highly selective antagonist at neuronal 5-hydroxytryptamine receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* 326: 36-44.

Fozard JR (1985) 5-Methoxytryptamine (5-MeOT) discriminates between excitatory neuronal 5-hydroxytryptamine (5-HT) receptors in the guinea-pig ileum. J. Pharmacol. 16: 498.

Fozard JR, Mobarok Ali ATM, Newgrosh G (1979) Blockade of serotonin receptors on autonomic neurons by (-)-cocaine and some related compounds. *Eur J Pharmacol* 59: 195-210.

Furness JB, Llewellyn-Smith IJ, Bornstein JC, Costa M (1988) Chemical neuroanatomy and the analysis of neuronal circuitry in the enteric nervous system. In: *Handbook of chemical neuroanatomy volume 6* (Björklund A, Hökfelt T, Owman C Eds.) Elsevier Amsterdam-New York-Oxford: pp. 161-218.

Gaddum JH, Picarelli ZP (1957) Two kinds of tryptamine receptor. *Br J Pharmacol Chemother* 12: 323-328.

Gale JD, Reeves JJ, Bunce KT (1993) Antagonism of the gastroprokinetic effect of metoclopramide by GR125487, a potent and selective 5-HT_4 receptor antagonist. *J Gastroint Motil* 5: 192P.

Gale JD, Grossman CJ, Whitehead JWF, Oxford AW, Bunce KT, Humphrey PPA (1994) GR113808: a novel, selective antagonist with high affinity at the 5-HT_4 receptor. *Br J Pharmacol* 111: 332-338.

Galligan JJ (1992) Differential inhibition of cholinergic and noncholinergic neurogenic contractions by 5-hydroxytryptamine$_{1A}$ receptor agonists in guinea pig ileum. *J Pharmacol Exp Ther* 260: 306-312.

Galligan JJ, North RA (1989) Drug receptors on single neurons. *Life Sci* 43: 2183-2192.

Galligan JJ, Surprenant A, Tonini M, North RA (1988) Differential localization of 5-HT_1 receptors on myenteric and submucosal neurons. *Am J Physiol* 255: G306-G611.

Gershon MD, Erde SM (1981) The nervous system of the gut. Gastroenterology 80: 1571-1594.

Gershon MD, Wade PR (1993) Enteric nervous system. *Curr Opinion Gastroenterol* 9: 246-253.

Gore S, Gilmore IT, Haigh CG, Morris AI (1989) Specific 5-hydroxytryptamine receptor (type 3) antagonist GR 38032F slows colonic transit. *Gut* 30: A713.

Gore S, Gilmore IT, Haigh CG, Brownless SM, Stockdale H, Morris AI (1990) Colonic transit in man is slowed by ondansetron (GR 38032F), a selective 5-hydroxytryptamine receptor (type 3) antagonist. *Aliment Pharmacol Ther* 4: 139-144.

Grossman CJ, Kilpatrick GJ, Bunce KT (1993) Development of a radioligand sinding assay for 5-HT_4 receptors in guinea-pig and rat brain. *Br J Pharmacol* 109: 618-624.

Gullikson GW, Loeffler RF, Viriña MA (1991) Relationship of serotonin-3 receptor antagonist activity to gastric emptying and motor-stimulating actions of prokinetic drugs in dogs. *J Pharmacol Exp Ther* 258: 103-110.

Gullikson GW, Virina MA, Loeffler RF, Yang DC, Goldstin, B, Flynn DL, Moormann AE (1992) Gastrointestinal motility responses to the S and R enantiomers of zacopride, a 5-HT_4 agonist and 5-HT_3 antagonist. *Drug Dev Res* 26: 405-417.

Gullikson GW, Virina MA, Loeffler RF, Yang DC, Goldstin B, Wang SX, Moummi C, Flynn DL, Zabrowski DL (1993) SC-49518 enhances gastric emptying of solid and liquid meals and stimulates gastrointestinal motility in dogs by a 5-hydroxytryptamine$_4$ receptor mechansim. *J Pharmacol Exp Ther* 264: 240-248.

Hasler WL, Heldsinger A, Owyang C (1991) Cisapride acts on muscarinic (glandular M_2) receptors to induce contraction of isolated gastric myocytes: mediation *via* a calcium-phosphoinositide pathway. *J Pharmacol Exp Ther* 259: 1294-1300.

Hay AM (1977) Pharmacological analysis of the effects of metoclopramide on the guinea pig isolated stomach. *Gastroenterology* 72: 864-869.

Hay AM, Man WK (1979) Effect of metoclopramide on guinea pig stomach. Critical dependence on intrinsic stores of acetylcholine. *Gastroenterology* 76: 492-496.

Heylen L, Luc G, Leysen JE (1992) In vitro receptor binding and neurotransmitter uptake inhibition profile of R 49 469 and R 51 619. Preclinical Research Report N 93195, Janssen Research Foundation.

Hillier K, Tam FS, Bunce KT, Grossman C (1994) Inhibition of motility induced by the activation of 5-HT_1-like and 5-HT_4-like receptors in isolated human colon smooth muscle. *Br J Pharmacol* 112 suppl.: 102P.

Hoyer D (1990) Serotonin 5-HT_3, 5-HT_4, and 5-HT-M receptors. *Neuropsychopharmacology* 3: 371-383.

Humphrey PPA, Feniuk W, Watts AD (1982) Ketanserin - a novel antihypertensive drug ? *J Pharm Pharmacol* 34: 541.

Jacoby HI, Brodie DA (1967) Gastrointestinal actions of metoclopramide. An experimental study. *Gastroenterology* 52: 676-684.

Justin-Besançon L, Laville C (1964) Action antiémétique du métoclopramide vis-à-vis de l'apomorphine et de l'hydergine. *C R Acad Sci* 158: 723-727.

Justin-Besançon L, Laville C, Thominet M (1964) Le métoclopramide et ses homologues. Introduction à leur étude biologique. *C R Acad Sci* t.258 (Groupe 13): 4384-4386.

Kadowaki M, Nagakura Y, Tomoi M, Mori J, Kohsaka M (1993) Effect of FK1052, a potent 5-hydroxytryptamine$_3$ and 5-hydroxytryptamine$_4$ receptor dual antagonist, on colonic function *in vivo*. *J Pharmacol Exp Ther* 266: 74-80.

Kaumann AJ, Sanders L, Brown AM, Murray KJ, Brown MJ (1990) A 5-hydroxytryptamine receptor in human atrium. *Br J Pharmacol* 100: 879-885.

Kidd E, Bouchelet de Vendegies I, Levy J-C, Hamon M, Gozlan H (1992) The potent 5-HT_3 receptor antagonist (R)-zacopride labels an additional high affinity site in the central nervous system. *Eur J Pharmacol* 221: 133-136.

Kidd E, Levy J-C, Nielsen M, Hamon M, Gozlan H (1993) Characterisation of the non-5-HT_3 high-affinity 'R' binding site for (R)-zacopride in brain and other tissues. *Eur J Pharmacol - Mol Pharmacol Sect* 247: 45-56.

Kilbinger H, Pfeuffer-Friederich I (1985) Two types of receptors for 5-hydroxytryptamine on the cholinergic nerves of the guinea-pig myenteric plexus. *Br J Pharmacol* 85: 529-539.

Kilbinger H, Wolf D (1992) Effects of 5-HT_4 receptor stimulation on basal and electrically evoked release of acetylcholine from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch Pharmacol* 345: 270-275.

Kilbinger H, Kruel R, Pfeuffer-Friederich I, Wessler I (1982) The effects of metoclopramide on acetylcholine release and on smooth muscle response in the isolated guinea-pig ileum. *Naunyn-Schmiedeberg's Arch Pharmacol* 319: 231-238.

Kilpatrick GJ, Bunce KT, Tyers MB (1990) 5-HT_3 receptors. *Med Res Rev* 10: 441-475.

King BF, Sanger GJ (1992) Facilitation of noncholinergic neurotransmission by renzapride (BRL 24924) in circular muscle of guinea-pig ileum. *Br J Pharmacol* 107 suppl.: 313P.

Kohli JD, Cripe LD (1979) Sulpiride: a weak antagonist of norepinephrine and 5-hydroxytryptamine. *Eur J Pharmacol* 56: 283-286.

Laduron PM, Leysen JE (1979) Domperidone, a specific *in vitro* dopamine antagonist, devoid of *in vivo* central dopaminergic ectivity. *Biochem Pharmacol* 28: 2161-2165.

Langlois M, Zhang L, Yang D, Brémont B, Shen S, Manara L, Croci T (1994) Design of a potent 5-HT_4 receptor agonist with nanomolar affinity. *Bioorg Med Chem Rev* 4: 1433-1436.

Leysen JE (1990) Gaps and peculiarities in 5-HT_2 receptor studies. *Neuropsychopharmacology* 3: 361-369.

Linnik MD, Butler BT, Gaddis RR, Ahmed NK (1991) Analysis of serotonergic mechansims underlying benzamide-induced gastroprokinesis. *J Pharmacol Exp Ther* 259: 501-507.

Longo WE, Vernava AM (1993) Prokinetic agents for lower gastrointestinal motility disorders. *Dis Colon Rectum* 36: 696-708.

Maayani S, Wilkinson CW, Stollak JS (1984) 5-hydroxytryptamine receptor in rabbit aorta: characterisation by butyrophenone analogs. *J Pharmacol Exp Ther* 229: 346-350.

Massingham R, Bou J, Roberts DJ (1985) A comparison of the stimulatory effects of metoclopramide and cinitapride in the guinea-pig isolated ileum. *J Auton Pharmacol* 5: 41-53.

Mawe GM, Branchek TA, Gershon MD (1986) Peripheral neural serotonin receptors: identification and characterization with specific antagonists and agonists. *Proc Natl Acad Sci USA* 83: 9799-9803.

Mawe GM, Branchek TA, Gershon MD (1989) Blockade of 5-HT-mediated enteric slow EPSPs by BRL 24924: gastrokinetic effects. *Am J Physiol* 257: G386-G396.

McRitchie B, McClelland CM, Cooper SM, Turner DH, Sanger GJ (1984) Dopamine antagonists as anti-emetics and as stimulants of gastric motility. In: *Mechanisms of gastrointestinal motility and secretion* (Bennett A, Velo G Eds.) New York, Plenum Press: pp287-301.

Meleagros L, Kreymann B, Ghatel MA, Bloom SR (1987) Mouth-to-caecum transit time in man is reduced by a novel serotonin M receptor antagonist. *Gut* 28 suppl.: A1373.

Meulemans AL, Schuurkes JAJ (1992) Is the action of cisapride on the guinea-pig ileum mediated *via* 5-HT_4 receptors? *Eur J Pharmacol* 212: 51-59.

Meulemans AL, Ghoos ECR, Cheyns P, Schuurkes JAJ (1995) 5-HT-induced relaxations on the human sigmoid colon are mediated via 5-HT_4 receptors. *Naunyn-Schmiedeberg's Arch Pharmacol*, in press (abstract)

Monferini E, Gaetani P, Rodriguez y Naena R, Giraldo E, Parenti M, Zocchetti A, Rizzi CA (1993) Pharmacological characterization of the 5-hydroxytryptamine receptor coupled to adenylyl cyclase stimulation in human brain. *Life Sci* 52: PL61-PL65.

Moriarty KJ, Higgs NB, Woodford M, Warhurst G, Turberg LA (1987) Inhibition of the effect of serotonin on rat ileal transport by cisapride: evidence in favour of the involvement of 5-HT_2 receptors. *Gut* 28: 844-848.

Moummi C, Yang D-C, Gullikson GW (1992) 5-HT_4 receptor activation induces relaxation and associated cAMP generation in rat esophagus. *Eur J Pharmacol* 216: 47-52.

Müller-Lissner SA (1987) Treatment of chronic constipation with cisapride and placebo. *Gut* 28: 1033-1038.

Nagakura Y, Kadowaki M, Tokoro K, Tomoi M, Mori J, Kohsaka M (1993) Pharmacological characterization of FK1052, a dihydropyridoindole derivative, as a new serotonin 3 and 4 dual receptor anatgonist. *J Pharmacol Exp Ther* 265: 752-758.

Nakayama S, Toshiaki N, Yamasato T, Takaki M, Itano N (1985) Effects of cisapride on the motility of digestive tract in dogs and guinea pigs. *Jpn J Smooth Muscle Res* 21: 1-9.

Nemeth PR, Gullikson GW (1989) Gastrointestinal motility stimulating drugs and 5-HT receptors on myenteric neurons. *Eur J Pharmacol* 166: 387-391.

Nemeth PR, Ort CA, Zafirov DH, Wood JD (1985) Interactions between serotonin and cisapride on myenteric neurons. *Eur J Pharmacol* 108: 77-83.

Nemeth PR, Palmer JM, Wood JD, Zafirov DH (1986) Effects of forskolin on electrical behaviour of myenteric neurones in guinea-pig small intestine. *J Physiol* 376: 439-450.

Nielsen OH, Hvid-Jacobsen K, Lund P, Langoholz E (1990) Gastric emptying and subjective symptoms of nausea: lack of effects of a 5-hydroxytryptamine-3 antagonist ondansetron on gastric emptying in patients with gastric stasis syndrome. *Digestion* 46: 89-96.

Niemegeers CJE, Schellekens KHL, Janssen PAJ (1980) The antiemetic effects of domperidone, a novel potent gastrokinetic. *Arch Int Pharmacodyn Ther* 244: 130-140.

North RA, Henderson G, Katayama Y, Johnson SM (1980) Electrophysiological evidence for presynaptic inhibition of acetylcholine release by 5-hydroxytryptamine in the enteric nervous system. *Neuroscience* 5: 581-586.

North RA, Slack BE, Surprenant A (1985) Muscarinic M_1 and M_2 receptors mediate depolarization and presynaptic inhibition in guinea-pig enteric nervous system. *J Physiol* 368: 435-452.

Ohno N, Itoh M, Yokoyama Y, Takeuchi T, Suzuki H (1993) Effects of cisapride on electrical and mechanical smooth muscles of the guinea-pig. *Gastroenterology* 104: A560.

Okwuasaba F, Hamilton JT (1976) The effect of metoclopramide on intestinal muscle responses and the peristaltic reflex *in vitro*. *Can J Physiol Pharmacol* 54: 393-404.

Palmer JM, Wood JD, Zafirov DH (1986) Elevation of adenosine 3',5'-phosphate mimicks slow synaptic excitation in myenteric neurons of the guinea-pig. *J Physiol* 376: 451-460.

Pan H, Galligan JJ (1994) 5-HT_{1A} and 5-HT_4 receptors mediate inhibition and facilitation of fast synaptic transmission in enteric neurons. *Am J Physiol* 266: G230-G238.

Paton WDM (1957) The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br J Pharmacol Chemother* 12: 119-127.

Peroutka SJ, Snyder SH (1979) Multiple serotonin receptors: Differential binding of [^{3}H]5-hydroxytryptamine, [^{3}H]lysergic acid diethylamide and [^{3}H]spiroperidol. *Mol Pharmacol* 16: 687-699.

Pfeuffer-Friederich I, Kilbinger H (1984) Facilitation and inhibition by 5-hydroxytryptamine and R51619 of acetylcholine release from guinea pig myenteric neurons. In: *Gastrointestinal motility*. Proceedings of the 9th International Symposium on Gastrointestinal Motility (Roman C Ed.) MTP Press, Lancaster UK, pp. 527-534.

Pinder RM, Brogden RN, Sawyer PR, Speight TM, Avery GS (1976) Metoclopramide: a review of its pharmacological properties and clinical use. *Drugs* 12: 81-131.

Ramirez MJ, Cenarruzabeitia E, Del Río J, Lasheras B (1994) Involvement of neurokinins in the non-cholinergic response to activation of 5-HT_3 and 5-HT_4 receptors in guinea-pig ileum. *Br J Pharmacol* 111: 419-424.

Reeves JJ, Bunce KT, Humphrey PPA (1991a) Investigation into the 5-hydroxytryptamine receptor mediating smooth muscle relaxation in the rat oesophagus. *Br J Pharmacol* 102: 1067-1072.

Reeves JJ, Gascoyne BH, Bunce KT (1991b) The gastroprokinetic benzamides increase gastric emptying in the rat through stimulation of 5-HT_4 receptors. *J Gastroint Motil* 3: P196.

Reynolds JC, Putnam PE (1992) Prokinetic agents. *Gastroenterol Clin North Am* 21: 567-596.

Reyntjens A, Verlinden M, Aerts T (1986) Development and clinical use of the new gastrointestinal prokinetic drug cisapride (R 51 619). *Drug Dev Res* 8: 251-265.

Richardson BP, Engel G, Donatsch P, Stadler PA (1985) Identification of serotonin M receptor subtypes and their specific blockade by a new class of drugs. *Nature* 316: 126-131.

Rizzi CA, Coccini T, Onori L, Manzo L, Tonini M (1992) Benzimidazolone derivatives: a new class of 5-hydroxytryptamine$_4$ receptor agonists with prokinetic and acetylcholine releasing properties in the guinea pig ileum. *J Pharmacol Exp Ther* 261: 412-419.

Rizzi CA, Sagrada A, Schiavone A, Schiantarelli P, Cesana R, Schiavi GB, Ladinsky H, Donetti A (1994) Gastroprokinetic properties of the benzimidazolone derivative BIMU 1, an agonist at 5-hydroxytryptamine$_4$ and antagonist at 5-hydroxytryptamine$_3$ receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* 349: 338-345.

Roberts DJ (1982) The pharmacological basis of the therapeutic activity of clebopride and related substituted benzamides. *Curr Ther Res* 31: S1-S44.

Sahyoun HA, Costall B, Naylor RJ (1982a) On the ability of domperidone to selectively inhibit catecholamine-induced relaxation of circular smooth muscle of guinea-pig stomach. *J Pharm Pharmacol* 34: 27-33.

Sahyoun HA, Costall B, Naylor RJ (1982b) Catecholamine-induced relaxation and contraction of the lower esophageal and pyloric sphincters of guinea-pig stomach: modification by domperidone. *J Pharm Pharmacol* 34: 318-324.

Sahyoun HA, Costall B, Naylor RJ (1982c) Benzamide action at α_2-adrenoceptors modifies catecholamine-induced contraction and relaxation of the circular smooth muscle from guinea-pig stomach. *Naunyn-Schmiedeberg's Arch Pharmacol* 319: 8-11.

Sanger GJ (1987a) Increased gut cholinergic activity and antagonism of 5-hydroxytryptamine M receptors by BRL 24924: potential clinical importance of BRL 24924. *Br J Pharmacol* 91: 77-87.

Sanger GJ (1987b) Activation of a myenteric 5-hydroxytryptamine-like receptor by metoclopramide. *J Pharm Pharmacol* 39: 449-453.

Sanger GJ, King FD (1988) From metoclopramide to selective gut motility stimulants and 5-HT_3 receptor antagonists. *Drug Des Del* 3: 273-295.

Sanger GJ, Nelson DR (1989) Selective and functional 5-hydroxytryptamine$_3$ receptor antagonism by BRL 43694 (granisetron). *Eur J Pharmacol* 159: 113-124.

Sanger GJ, Wardle KA, Shapcott S, Yee KF (1991) Constipation evoked by 5-HT_3 receptor antagonists. In: *Serotonin: Molecular biology, receptors and functional effects* (Fozard JR, Saxena PR Eds.) Birkhäuser Verlag, Basel, pp. 381-388.

Schemann M (1991) 5-Hydroxytryptamine-mediated responses in Myenteric neurons of the guinea-pig gastric corpus: effect of ICS 205-930 and cisapride. *J Gastroint Motil* 3: 255-262.

Schiavi GB, Brunet S, Rizzi CA, Ladinsky H (1994) Identification of serotonin 5-HT_4 recognition sites in the porcine caudate nucleus by radioligand binding. *Neuropharmacology* 33: 543-549.

Schiavone A, Volonté M, Micheletti R (1990) The gastrointestinal motor effects of benzamide derivatives is unrelated to 5-HT_3 receptor blockade. *Eur J Pharmacol* 187: 323-329.

Schultz R, Cartwright C (1974) Effect of morphine on serotonin release from the myenteric plexus of the guinea-pig. *Br J Pharmacol* 12: 115-118.

Schulze-Delrieu K (1979) Metoclopramide. *Gastroenterology* 77: 768-779.

Schuurkes JAJ (1992) Gastrointestinal motility effects of cisapride mediated by nonadrenergic, noncholinergic nerves. In: *Gastrointestinal dysmotility: focus on cisapride* (Heading RC, Wood JD Eds.) Raven Press, New York, pp. 1-14.

Schuurkes JAJ, Van Nueten JM (1981) Effects of dopamine and its antagonist domperidone cannot be explained by an effect on α_1-adrenergic receptors. *Arch Int Pharmacodyn Ther* 250: 324-327.

Schuurkes JAJ, Van Nueten JM (1984) Domperidone improves myogenically transmitted antroduodenal coordination by blocking dopaminergic receptor sites. *Scand J Gastroenterol* suppl. 96: 101-110.

Schuurkes JAJ, Van Nueten JM (1988) 5-HT_3 serotonergic mechanisms and stimulation of gastrointestinal motility by cisapride. *Gastroenterology* 94: A415.

Schuurkes JAJ, Helsen LFM, Van Nueten JM (1982) Improved gastroduodenal coordination by the peripheral dopamine antagonist domperidone. In: *Motility of the digestive tract* (Wienbeck Ed.), Raven Press,New York: pp. 565-572.

Schuurkes JAJ, Helsen LFM, Van Nueten JM (1985a) A comparative study on the effects of domperidone, metoclopramide, clebopride and trimebutine on the gastro-duodenal preparation of the guinea-pig. *Jap J Pharmacol* 39: 123-130.

Schuurkes JAJ, Van Nueten JM, Van Daele PGH, Reyntjens AJ, Janssen PAJ (1985b) Motor stimulating properties of cisapride on isolated gastrointestinal preparations of the guinea-pig. *J Pharmacol Exp Ther* 234: 775-783.

Schuurkes JAJ, Megens AAHP, Niemegeers CJE, Leysen JE (1987) A comparative study of the cholinergic vs the anti-dopaminergic properties of benzamides with gastrointestinal prokinetic activity. In: *Cellular physiology and clinical studies of gastrointestinal smooth muscle* (Szurszewski JH, Ed.) Elsevier Amsterdam-New York-Oxford: pp. 231-247.

Schuurkes JAJ, Van Bergen PJE, Van Nueten JM (1988) Prejunctional muscarinic (M_1) receptor interactions on guinea-pig ileum: lack of effect of cisapride. *Br J Pharmacol* 94: 228-234.

Schuurkes JAJ, Meulemans AL, Obertop H, Akkermans LMA (1991) 5-HT_4 receptors on the human stomach. *J Gastroint Motil* 3: P199.

Shenker A, Maayani S, Weinstein H, Green JP (1987) Pharmacological characterization of two 5-hydroxytryptamine receptors coupled to adenylate cyclase in guinea-pig hippocampal membranes. *Mol Pharmacol* 31: 357-367.

Shepperson NB, Duval N, Massingham R, Langer SZ (1982) Differential blocking effects of several dopamine receptor antagonists for peripheral pre- and postsynaptic dopamine receptors in the anaestetized dog. *J Pharmacol Exp Ther* 221: 753-761.

Spedding M (1980) Effects of metoclopramide and isoprenaline in the rat vas deferens; interactions with α-adrenoceptors.*Br J Pharmacol* 71: 113-120.

Stacher G, Gaupmann G, Schneider C, tacher Janotta G, Steiner-Mittelbach G, Abatzi T, Steinringer H (1989) Effects of a 5-hydroxytryptamine$_3$ receptor antaginist (ICS 205-930) on colonic motor activity in healthy men. *Br J Clin Pharmacol.* 28: 315-322.

Stacher G, Bergmann H, Schneider C, Steiner-Mittelbach G, Gauptmann G, Steinringer H, Abatzi TA, Stacher-Janotta G (1990) Effects of 5-HT_3 receptor antagonist ICS 205-930 on fat-delayed gastric emptying and antral motor activity. *Br J Clin Pharmacol* 30: 41-48.

Tack JF, Janssens J, Vantrappen G, Wood JD (1992) Actions of 5-hydroxytryptamine on myenteric neurons in guinea pig gastric antrum. *Am J Physiol* 263: G838-G846.

Takaki M, Branchek T, Hadassah T, Gershon MD (1985) Specific antagonism of enteric neural serotonin receptors by dipeptides of 5-hydroxytryptophan: evidence that serotonin is a mediator of slow synaptic excitation in the myenteric plexus. *J Neurosci* 5: 1769-1780.

Takeda K, Taniyama K, Kuno, T, Ishikawa T, Ohmura I, Tanaka C (1991) Clebopride enhances contractility of the guinea-pig stomach by blocking peripheral D_2 dopamine receptor and alpha-2 adrenoceptor. *J Pharmacol Exp Ther* 257: 806-811.

Talley NJ, Phillips SF, Haddad A, Miller LJ, Twomey C, Zinsmeister AR, Ciociola A (1989) Effect of selective 5-HT_3 antagonist (GR 38032F) on small bowel transit and release of gastrointestinal peptides. *Dig Dis Sci* 34: 1511-1515.

Talley NJ, Phillips SF, Haddad A, Miller LJ, Twomey C, Zinsmeister AR, MacCarty RL, Ciociola A (1990) GR 38032F (Ondansetron), a selective 5HT_3 receptor antagonist, slows colonic transit in healthy man. *Dig Dis Sci* 35: 477-480.

Taludhar BR, Costall B, Naylor RJ (1991) Putative 5-HT_4 receptor involvement in the relaxations induced by 5-HT in the rat ileum. *Br J Pharmacol* 104 suppl.: 151P.

Taludhar BR, Costall B, Naylor RJ (1992) Variation in the relaxation response to 5-HT in different regions of the rat small intestine. *Br J Pharmacol* 105 suppl.: 278P.

Taludhar BR, Costall B, Naylor RJ (1994a) Pharmacological characterization of the relaxation response to 5-HT in the rat terminal ileum using the 5-HT_4 receptor antagonist GR 113808. *Br J Pharmacol* 112 suppl: 172P.

Taludhar BR, Costall B, Naylor RJ (1994b) Differential activity of (R) and (S)-zacopride to modify 5-HT_4 receptor mediated relaxation of the rat terminal ileum. *Br J Pharmacol* 112 suppl: 173P.

Tam FSF, Hillier K, Bunce KT (1994) Characterization of the 5-hydroxytryptamine receptor type involved in inhibition of spontaneous activity of human isolated colonic circular muscle. *Br J Pharmacol*, 113: 143-150.

Taniyama K, Nakayama S, Takeda K, Matsuyama S, Shirakawa J, Sano I, Tanaka C (1991) Cisapride stimulates motility of the intestine *via* the 5-hydroxytryptamine receptors. *J Pharmacol Exp Ther* 258: 1098-1104.

Tonini M, Galligan JJ, North RA (1989) Effects of cisapride on cholinergic neurotransmission and propulsive motility in the guinea pig ileum. *Gastroenterology* 96: 1257-1264.

Tonini M, Rizzi CA, Manzo L, Onori L (1991) Novel enteric 5-HT_4 receptors and gastrointestinal prokinetic action. *Pharmacol Res* 24: 5-14.

Tonini M, Candura SM, Onori L, Coccini T, Manzo L, Rizzi CA (1992) 5-Hydroxytryptamine$_4$ receptor agonists facilitate cholinergic transmission in the circular muscle of guinea pig ileum: antagonism by tropisetron and DAU 6285. *Life Sci* 50: PL 173-PL 178.

Trendelenburg P (1917) Physiologische und pharmakologische Versuche über die Dünndarmperistaltik. *Naunyn-Schmiedeberg's Arch Exp Pathol Pharmakol* 81: 55-129.

Valdovinos MA, Thomforde GM, Camilleri M (1993) Effect of putative carcinoid mediators on gastric and small bowel transit in rats and the role of 5-HT receptors. Aliment Pharmacol Ther 7: 61-66.

Van Nueten JM, Ennis C, Helsen L, Laduron PM, Janssen PAJ (1978) Inhibition of dopamine receptors in the stomach: an explanation of the gastrokinetic properties of domperidone. *Life Sci* 23: 453-458.

Van Wijngaarden I, Tulp MTM, Soudijn W (1990) The concept of selectivity in 5-HT receptor research. *Eur J Pharmacol-Mol Pharmacol Sect* 188: 301-312.

Wade PR, Gershon MD (1991) Subtypes of 5-HT receptor in the peristaltic reflex of the guinea pig. *J Gastroint Motil* 3: P206.

Wade PR, Mawe GM, Branchek TA, Gershon MD (1991) Use of stereoisolers of zacopride to analyze actions of 5-hydroxytryptamine on enteric neurons. *Am J Physiol* 260: G80-G90.

Wade PR, Tamir H, Kirckgessner AL, Gershon MD (1994) Analysis of the role of 5-HT in the enteric nervous system using anti-idiotopic antibodies to 5-HT receptors. *Am J Physiol* 266: G403-G416.

Wardle KA, Sanger GJ (1993) The guinea-pig distal colon - a sensitive preparation for the investigation of 5-HT_4 receptor-mediated contractions. *Br J Pharmacol* 110: 1593-1599.

Wardle KA, Ellis ES, Baxter GS, Kennett GA, Gaster LM, Sanger GJ (1994) The effects of SB 204070, a highly potent and selective 5-HT_4 receptor antagonist, on the guinea-pig distal colon. *Br J Pharmacol* 112: 789-794.

Willems JL, Buylaert WA, Lefebvre RA, Bogaert MG (1985) Neuronal dopamine receptors on autonomic ganglia and sympathetic nerves and dopamine receptors in the gastrointestinal system. *Pharmacol Rev* 37: 165-216.

Wilmer A, Tack J, Coremans G, Janssens J, Peeters T, Vantrappen G (1993) *Gastroenterology* 105: 773-780.

Wiseman LR, Faulds D (1994) Cisapride. An updated review of its pharmacology and therapeutic efficacy as a prokinetic agent in gastrointestinal motility disorders. *Drugs* 47: 116-152.

Wood JD (1994) Application of classification schemes to the enteric nervous system. *J Auton Nerv Syst* 48: 17-29.

Yau WM, Youther ML (1993) 5-HT_4 receptor on myenteric cholinergic neurons involved cAMP and K^+ channel. *J Gastroint Motil* 5: 226P.

Yoshida N, Ito T, Karasawa T, Itoh Z (1991a) AS-4370, a new gastrokinetic agent, enhances upper gastrointestinal motor activity in conscious dogs. *J Pharmacol Exp Ther* 257: 781-787.

Yoshida N, Mizumoto A, Iwanaga Y, Itoh Z (1991b) Effects of 5-hydroxytryptamine$_3$ receptor antagonists on gastrointestinal motor activity in conscious dogs. *J Pharmacol Exp Ther* 256: 272-278.

Chapter 2

Pharmacological characterization of 5-hydroxytryptamine receptor types in the guinea-pig proximal colon

M.R. Briejer, L.M.A. Akkermans, A. L. Meulemans,
R.A. Lefebvre and J.A.J. Schuurkes

slightly modified: *J Gastroint Motil* (1993) 5: 239-246

Abstract. It was investigated which 5-hydroxytryptamine (5-HT) receptors mediate the responses to 5-HT in the longitudinal muscle layer of the guinea-pig proximal colon, using selective 5-HT receptor antagonists and the 5-HT analogues α-methyl-5-HT (α-Me-5-HT), 2-methyl-5-HT (2-Me-5-HT), and 5-methoxytryptamine (5-MeOT).

5-HT as well as its analogues induced concentration-related contractions, at low concentrations preceded by relaxations. The 5-HT concentration-contractile response curve was biphasic whilst the curves to α-Me-5-HT, 2-Me-5-HT, and 5-MeOT were monophasic. Tetrodotoxin (TTX) abolished the relaxations, and it inhibited the contractions to all agonists. In the presence of TTX, blockade of either 5-HT_2 (ketanserin) or 5-HT_3 receptors (ondansetron, tropisetron) reduced the contractions to 5-HT, whereas blockade of both 5-HT receptor types at the same time abolished them. In the absence of TTX, the contractions to 5-HT were inhibited by antagonists at 5-HT_2 (ketanserin), 5-HT_3 (granisetron, nanomolar concentration of tropisetron) and also 5-HT_4 receptors (micromolar concentration of tropisetron). Contractions to α-Me-5-HT did not seem to be sensitive to 5-HT_2 receptor blockade with ketanserin, but in the presence of TTX the contractions were abolished by the 5-HT_2 receptor antagonist. The 5-HT_3 receptor antagonist granisetron abolished contractions to 2-Me-5-HT. In the presence of TTX, the 5-HT_2 receptor antagonist ketanserin abolished contractions to 5-MeOT, and in the absence of TTX the contractions to 5-MeOT were highly sensitive blockade of 5-HT_4 receptors with tropisetron. Blockade of either 5-HT_1 (methiothepin), 5-HT_2 (ketanserin), 5-HT_3 (ondansetron, granisetron, tropisetron) or 5-HT_4 (tropisetron) receptors did not abolish the relaxations to 5-HT or 5-MeOT.

Conclusions: 5-HT induces contractions of the longitudinal muscle of the guinea-pig proximal colon, through the stimulation of 5-HT_2 receptors on the smooth muscle cells and 5-HT_3 receptors and putative neuronal 5-HT_4 receptors. 5-HT evokes relaxations *via* an unknown neuronal receptor.

Introduction

5-Hydroxytryptamine (5-HT) is stored in high concentrations in the enterochromaffin cells of the mucosa, from which it might be released by applying pressure to the mucosa. 5-HT is a neurotransmitter in the enteric nervous system, and is involved directly and indirectly in the regulation of motility (Gershon & Erde, 1981; Furness *et al.*, 1988; Gershon *et al.*, 1989). In the guinea-pig proximal colon, the presence of 5-HT has been immunohistochemically demonstrated in neurons of the myenteric plexus (Legay *et al.*, 1984). In colonic preparations, 5-HT induces both relaxations and contractions (Costa & Furness, 1979; Kojima & Shimo, 1986), but it is not known which receptors mediate these responses to 5-HT. Many types of 5-HT receptors have been demonstrated to exist in different tissues of several animal species and man. A classification of 5-HT receptors into 5-HT_1, 5-HT_2 and 5-HT_3 receptors is established (Bradley *et al.*, 1986). Recently, Dumuis and co-workers (Dumuis *et al.*, 1988), from their work on mouse embryo colliculi neurons, proposed to add yet another, new 5-HT-receptor type to the 5-HT receptor classification scheme, logically to be designated 5-HT_4 receptor (Bockaert *et al.*, 1992).

The aim of this study was to gain more insight into which types of 5-HT receptor are involved in the action of 5-HT on the guinea-pig proximal colon. We therefore investigated the responses to 5-HT and its analogues, α-methyl-5-HT (α-Me-5-HT, 5-HT_2 receptor agonist), 2-methyl-5-HT (2-Me-5-HT, 5-HT_3 receptor agonist), and 5-methoxytryptamine (5-MeOT, 5-HT_4 receptor agonist), in the absence and presence of various selective 5-HT receptor antagonists and/or tetrodotoxin (TTX) to eliminate nerve-mediated responses (Gershon, 1967).

Methods and materials

Tissue preparation

Dunkin-Hartley guinea-pigs of either sex, weighing 400-600 g, were killed by decapitation. The proximal colon was removed, and the luminal contents were washed out with De Jalon's solution (composition in mM: KCl 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, NaCl 155, glucose 2.78). Starting at the proximal end, about 1 cm distal to the caecum, the colon was divided into 4 segments of 3 cm. These intact segments were individually mounted vertically into an organ bath containing 20 ml De Jalon's solution for isotonic measurement of longitudinal muscle responses. This solution was kept at 37 °C and gassed with carbogen (95% O_2, 5% CO_2). The strips were subjected to a preload of 2 g and allowed to stabilize for half an hour. After stabilization, the contraction to methacholine (0.3 μM) was measured. The measurement was repeated after washing (i.e. replacing the bathing fluid twice) and stabilization until the response to methacholine remained constant (usually 3 times). The mean of the last two responses was taken as 100%. The bathing fluid was replaced after 30 min at most.

Establishment of concentration-response curves

Agonists and antagonists were applied directly to the organ baths. Non-cumulative concentration-response curves were constructed with a 15 min dosing cycle. Agonist concentration series 10 nM, 30 nM, 0.1 μM, 0.3 μM, etc. were applied in ascending order. The preparations were preincubated with antagonists for 15 min, thus fitting into the 15 min agonist dosing cycle. The maximum amplitude of the contractions was measured, and agonists were washed out as soon as the peak responses were reached; contact with the tissue was 2 min at most. When biphasic responses occurred (relaxation preceding a contraction), the maximal contraction was measured from the base line (before the agonist was added) to the highest point of contraction, even if the contraction height was

negative as compared to baseline. This manner of quantification of contractions was chosen since the relaxatory component in the action of 5-HT was variable between different preparations. All measurements were expressed as a percentage of the methacholine-induced contractions. For each preparation, only one concentration-response curve was established.

Compounds

Solutions of ketanserin tartrate, 2-methyl-5-hydroxytryptamine tartrate (Janssen Pharmaceutica, Belgium), 5-methoxytryptamine HCl (Janssen Chimica, Belgium), α-methyl-5-hydroxytryptamine maleate (Cookson Chemicals Ltd., UK), methiothepin maleate (Hoffman-La Roche, Switzerland), methacholine HCl (E.Merck, Germany), tropisetron HCl (Sandoz, Switzerland), ondansetron (Glaxo, United Kingdom), granisetron HCl (Beecham, United Kingdom), 5-hydroxytryptamine HCl and tetrodotoxin (Serva, Germany) were added to the organ bath solution in volumes less than 1% of the bath volume. All compounds were dissolved in distilled water, except for 5-HT (stock containing ascorbic acid (0.25 μM). The vehicle had no effect on the tone of the muscle. None of the above antagonists had an intrinsic effect, except for TTX (0.3 μM) and tropisetron (3 μM), which caused contractions of about 19 % and 6 % respectively of (0.3 μM) methacholine-induced contractions. None of the antagonists inhibited the contractions to methacholine (0.3 μM), except for tropisetron (3 μM), which caused a significant ($P < 0.05$) inhibition of about 10 %. The stock solution of TTX was prepared in advance and kept frozen (-25 °C) in aliquots of 0.1 mM.

Statistical analysis

For graphical representation means ± standard error of the means were calculated. Differences between mean values were tested with one way analysis of variance using the Scheffé F-test (comparison treatment vs. treatment) or Dunnett's t-test (treatment vs. control) (Wallenstein *et al.*, 1980). The level of significance was set at $P < 0.05$.

Results

5-Hydroxytryptamine

5-HT concentration-dependently induced both relaxations and contractions (10 nM - 30 μM) as exemplified in Figure 2.1. The observed relaxations were

variable in magnitude (but always less than 20 % of the methacholine-induced contractions), and they were masked by the contractions in the higher 5-HT concentration region (Figure 2.1). The concentration-response curve for the contractile effect of 5-HT appeared biphasic (Figure 2.2) with a maximum effect of 112 ± 7 % at 30 μM 5-HT (n = 12).

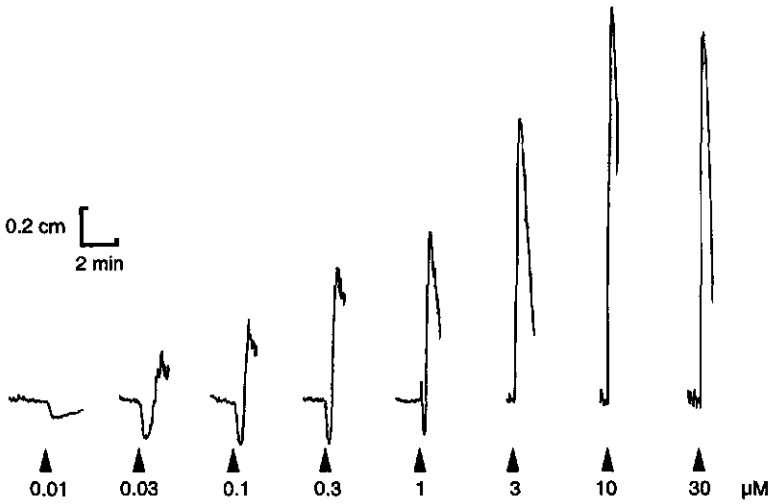


Figure 2.1 Tracing of responses induced by increasing concentrations of 5-hydroxytryptamine. 5-Hydroxytryptamine induced contractions as well as relaxations in the lower concentration region, but only contractions in the high concentration region.

TTX (0.3 μM) greatly affected the responses to 5-HT (n = 6). In the presence of TTX, 5-HT-induced relaxations were abolished (not shown). TTX reduced the contractile response to 5-HT from 30 nM onwards (Figure 2.2). TTX 1 μM and TTX 3 μM reduced the contractions to 5-HT 1 μM to a similar extent as did TTX 0.3 μM (n = 6, results not shown), indicating that 0.3 μM TTX is sufficient to eliminate TTX-sensitive responses. In the presence of TTX (0.3 μM), ondansetron (3 μM; n = 6) and tropisetron (0.3 μM; n = 6) reduced the contractions to 5-HT (> 3 μM) even more as compared to the inhibition by TTX alone (Figure 2.2, *left panel*). In the presence of TTX, ketanserin (0.3 μM; n = 6) inhibited 5-HT-induced contractions completely at concentrations of 5-HT ≤ 1 μM. At concentrations of 5-HT > 3 μM, the 5-HT-induced responses were inhibited by ketanserin, as compared to the TTX control curve (Figure 2.2, *right*

panel). In the presence of TTX, ketanserin (0.3 μM) in combination with tropisetron (0.3 μM; n = 6) (Figure 2.2, *right panel*) or granisetron (0.3 μM; n = 4, not shown) abolished the 5-HT-induced responses.

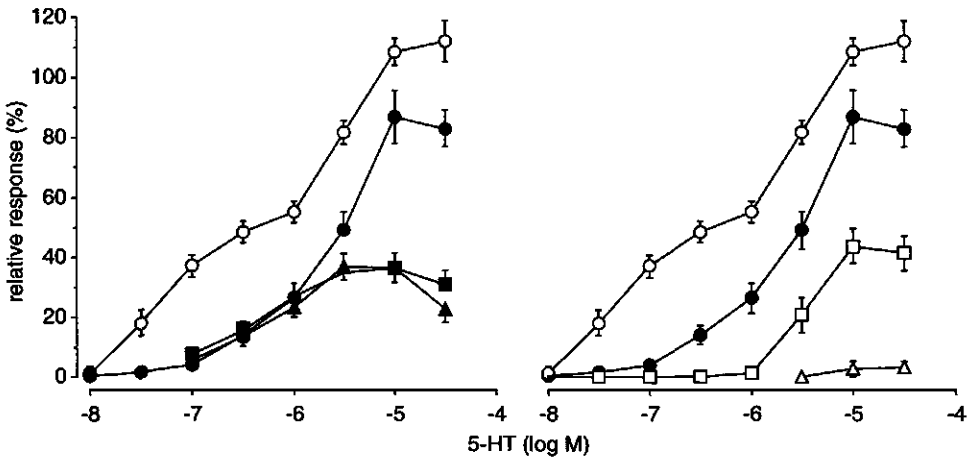


Figure 2.2 The concentration-response curve to 5-HT in the absence of antagonists (O), and in the presence of TTX (0.3 μM) (●), TTX (0.3 μM) + ondansetron (3 μM) (■), TTX (0.3 μM) + tropisetron (0.3 μM) (▲), TTX (0.3 μM) + ketanserin (0.3 μM) (□), and a combination of TTX (0.3 μM), ketanserin (0.3 μM) and tropisetron (0.3 μM) (Δ). All data were expressed as mean values ± s.e.m (n = 4-12) relatively to methacholine (0.3 μM)-induced responses.

Tropisetron and granisetron (both 0.3 μM; n = 6-8) significantly ($P < 0.05$) reduced the contractions to 5-HT > 1 μM (i.e. the second phase of the concentration-response curve, Figure 2.3, *left panel*). At a concentration of 3 μM tropisetron (n = 6), the contractions to 5-HT were even further reduced (Figure 2.3, *left panel*). The inhibition of the 5-HT-induced contractions by tropisetron 3 μM was significantly ($P < 0.05$) different from that by tropisetron 0.3 μM up to 10 μM 5-HT. Ketanserin (0.3 μM; n = 6) depressed the concentration-response curve of 5-HT at concentrations of 5-HT ≥ 0.1 μM (Figure 2.3, *right panel*). When the strips were preincubated with both ketanserin (0.3 μM) and granisetron (0.3 μM) (n = 6), 5-HT-induced contractions were inhibited at 10 μM 5-HT as compared to the contractions in the presence of ketanserin alone. A combination of ketanserin (0.3 μM), granisetron (0.3 μM) and tropisetron (3 μM) (n = 4) inhibited 5-HT-induced responses from 30 nM onwards as compared to 5-HT control (Figure 2.3, *right panel*). The inhibition of the contractions to 5-HT in the

range 0.1 μM - 1 μM by the combination of ketanserin, granisetron and tropisetron differed significantly ($P < 0.05$) from the inhibition by the combination of ketanserin and granisetron without tropisetron. Neither one of the antagonists mentioned above, nor methiothepin (0.1 μM; n = 6) abolished the relaxations that were induced by 5-HT (not shown).

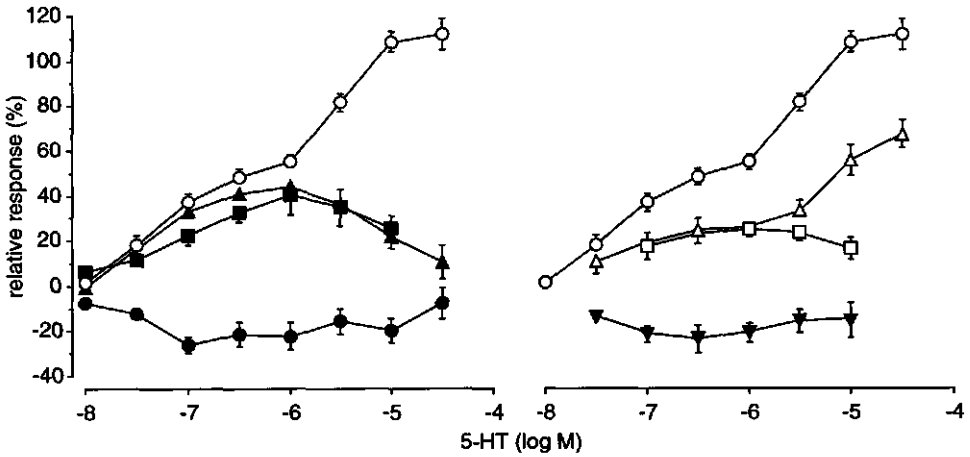


Figure 2.3 The concentration-response curve to 5-HT in the absence of antagonists (O), and in the presence of granisetron (0.3 μM) (■), tropisetron (0.3 μM) (▲) and (3 μM) (●), ketanserin (0.3 μM) (Δ), ketanserin (0.3 μM) + granisetron (0.3 μM) (□), and a combination of ketanserin (0.3 μM), granisetron (0.3 μM) and tropisetron (3 μM) (▼). All data were expressed as mean values ± s.e.m (n = 4-12) relatively to methacholine (0.3 μM)-induced responses.

α-Methyl-5-hydroxytryptamine

(Figure 2.4) α-Me-5-HT induced both relaxations and contractions from 0.1 μM onwards. The concentration-response curve for the contractile effect of α-Me-5-HT appeared monophasic (n = 6). Again, TTX (0.3 μM; n = 6) abolished the relaxant component of the effect of α-Me-5-HT, but it only significantly ($P < 0.05$) inhibited the contractions to 10 μM α-Me-5-HT. Ketanserin (0.3 μM; n = 6) did not significantly ($P > 0.05$) affect the contractions to α-Me-5-HT. However, in the presence of TTX, ketanserin (n = 6) abolished the responses to α-Me-5-HT (significant at 3 μM and 10 μM as compared to control, significant at 10 μM as compared to the contractions in the presence of TTX ($P < 0.05$). Neither one of the antagonists mentioned above abolished the relaxations that were induced by α-Me-5-HT (not shown).

2-Methyl-5-hydroxytryptamine

(Figure 2.5) 2-Me-5-HT induced both relaxations and contractions from 3 μM onwards. The concentration-response curve of the contractile effect of 2-Me-5-HT appeared monophasic, with a maximum at 30 μM (n = 4). TTX (n = 4) abolished the relaxations, and it inhibited the contractions to 2-Me-5-HT from 10 μM onwards. Granisetron (0.3 μM; n = 4) alone and in the presence of TTX (n = 4), abolished the contractions to 2-Me-5-HT (significantly ($P < 0.05$) as compared to control from 10 μM onwards, as compared to the contractions in the presence of TTX at 10 μM and 30 μM). Neither one of the antagonists mentioned above abolished the relaxations that were induced by 2-Me-5-HT (not shown).

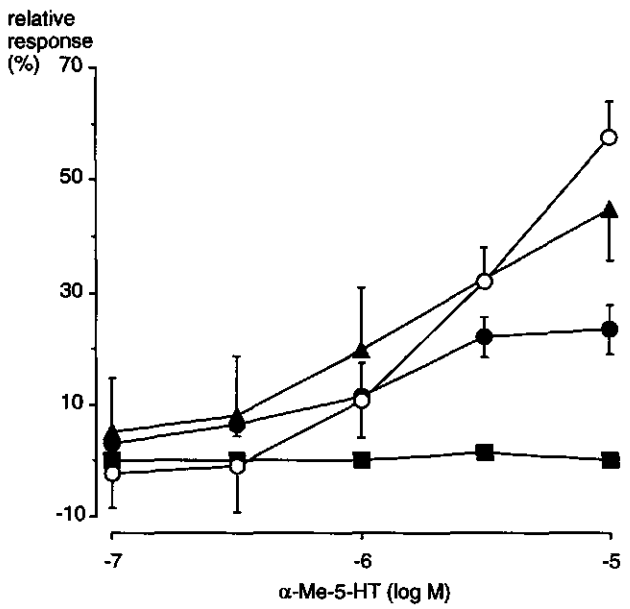


Figure 2.4 The concentration-response curve to α-Me-5-HT in the absence of antagonists (○), and in the presence of ketanserin (0.3 μM) (▲), TTX (0.3 μM) (●), and TTX (0.3 μM) + ketanserin (0.3 μM) (■). All data were expressed as mean values ± s.e.m (n = 6) relatively to methacholine (0.3 μM)-induced responses.

5-Methoxytryptamine

(Figure 2.6) Like the other tryptamines tested, 5-MeOT induced both relaxations and contractions from 30 nM onwards. The concentration-response curve for the contractile effect of 5-MeOT appeared monophasic, the maximum

response occurred at 3 μM 5-MeOT (47 ± 3 %; n = 12). TTX (0.3 μM; n = 14) abolished the relaxations and inhibited the contractions to 5-MeOT. The concentration-response curve to 5-MeOT in the presence of TTX 0.3 μM coincided with that in the presence of TTX 1 μM (n = 6; not shown). In the presence of TTX, tropisetron (3 μM; n = 6) did not significantly ($P > 0.05$) inhibit the contractions to 5-MeOT any further as compared to the TTX curve (Figure 2.6, *left panel*). However, in the absence of TTX, tropisetron (n = 6) strongly inhibited the contractions to 5-MeOT (Figure 2.6, *left panel*). Ketanserin (0.3 μM; n = 6) inhibited the contractions to 5-MeOT in the concentration range 1 - 10 μM (Figure 2.6, *right panel*). In the presence of TTX, ketanserin (n = 4) abolished the contractions to 5-MeOT (Figure 2.6, *right panel*). Neither one of the above mentioned antagonists, nor methiothepin (0.1 μM), abolished the 5-MeOT-induced relaxations.

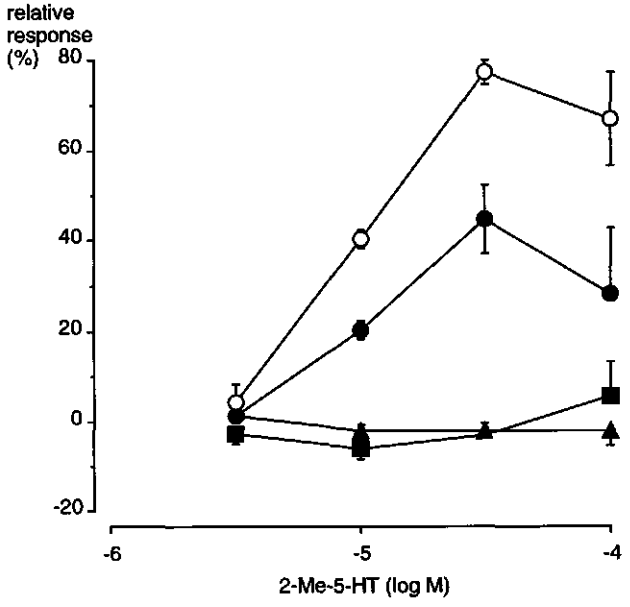


Figure 2.5 The concentration-response curve to 2-Me-5-HT in the absence of antagonists (O), and in the presence of granisetron (0.3 μM) (■), TTX (0.3 μM) (●), and TTX (0.3 μM) + granisetron (0.3 μM) (▲). All data were expressed as mean values ± s.e.m (n = 4) relatively to methacholine (0.3 μM)-induced responses.

Discussion

The role and function of 5-HT in the gastrointestinal tract is still poorly understood. This study on the action of 5-HT on the guinea-pig proximal colon shows that 5-HT displays a complex pattern of action.

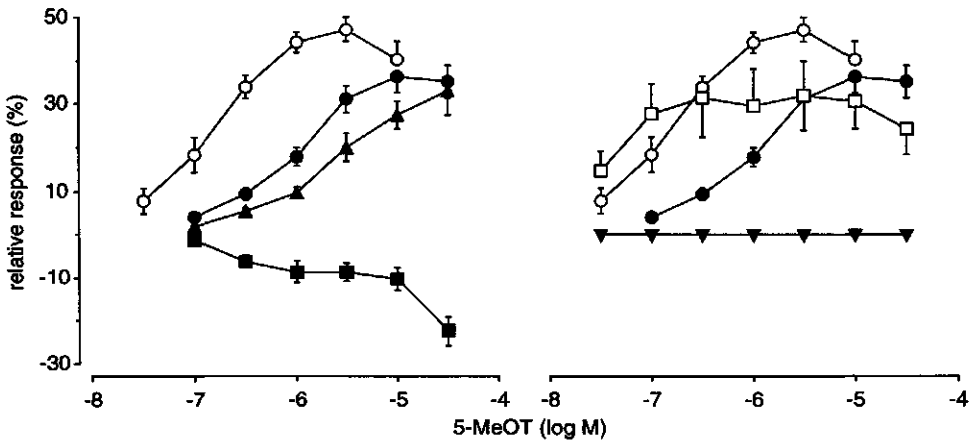


Figure 2.6 The concentration-response curve to 5-MeOT in the absence of antagonists (O), and in the presence of TTX (0.3 μM) (●), tropisetron (3 μM) (■), TTX (0.3 μM) + tropisetron (3 μM) (▲), ketanserin (0.3 μM) (□), and TTX (0.3 μM) + ketanserin (0.3 μM) (▼). All data were expressed as mean values ± s.e.m (n = 4-14) relatively to methacholine (0.3 μM)-induced responses.

The observed relaxations, elicited by the tryptamine analogues, could not be inhibited by the following 5-HT-receptor antagonists at the receptor types: 5-HT_1 (methiothepin), 5-HT_2 (ketanserin, methiothepin), 5-HT_3 (granisetron, ondansetron, tropisetron 0.3 μM) or 5-HT_4 (tropisetron 3 μM [Bockaert *et al.*, 1992]). Other investigators observed 5-HT-induced relaxations in their preparation of the longitudinal muscle of the guinea-pig proximal and distal colon as well, which could not be blocked with the 5-HT_1-receptor antagonist methysergide (up to 2.8 μM) (Costa & Furness, 1979; Onori *et al.*, 1984; Kojima & Shimo, 1986). Other workers (Kojima & Shimo, 1986; Kojima, 1991), however, studied the 5-HT-induced relaxations of the guinea-pig proximal colon in more detail, and suggested that the relaxations are mediated through 5-HT_1 receptors. The exact

5-HT-receptor subtype *via* which the relaxations are evoked, needs further investigation.

The neurotoxin tetrodotoxin (TTX) eliminates responses mediated by nervous structures, as it blocks Na^+ channels involved in the propagation of action potentials along the axons. Responses to drugs that act directly on the smooth muscle are unaffected by TTX (Gershon, 1967). TTX abolished the relaxations that were induced by either of the tryptamines, suggesting neuronal involvement, which confirms observations of other investigators (Kojima & Shimo, 1986; Kojima, 1991; Elswood & Bunce, 1992). Furthermore, in another study we have shown that 5-HT induces relaxations through the release of the inhibitory putative transmitter nitric oxide (NO) from the intrinsic nerves (Briejer *et al.*, 1992).

The contractions elicited by 5-HT appear to be mediated by several 5-HT receptor types, illustrated by the fact that the concentration-response curve of 5-HT is biphasic. TTX inhibited the 5-HT-induced contractions to a considerable extent, corresponding to the observations of other investigators (Costa & Furness, 1979; Onori *et al.*, 1984; Kojima & Shimo, 1986). These observations suggest that 5-HT acts partly *via* the enteric neurons, and partly directly on the smooth muscle cells.

Tropisetron, ondansetron and granisetron are established selective 5-HT_3 receptor antagonists (Brittain, 1987; Sanger *et al.*, 1988; Sanger & Nelson, 1989). 2-Me-5-HT is a selective 5-HT_3 receptor agonist without appreciable activity at 5-HT_2 or 5-HT_4 receptors (Bradley *et al.*, 1987; Craig *et al.*, 1990; Bockaert *et al.*, 1992). However, it has activity at the rabbit renal artery 5-HT_1-like receptor (Tadipatri & Saxena, 1992), and in our preparation it induced relaxations, which might also be evoked through 5-HT_1 receptor stimulation (see above). Granisetron abolished the contractile response to 2-Me-5-HT. Granisetron, ondansetron and tropisetron (0.3 μM) also antagonized the responses to 5-HT. Hence, 5-HT_3 receptors are present in the guinea-pig proximal colon, as they are in the distal colon (Butler *et al.*, 1990). Since the 5-HT_3 receptor-mediated contractions were in part TTX-sensitive, the 5-HT_3 receptors are located, at least in part, on neurons. The observed inhibition by 5-HT_3 receptor antagonists of the contractions to 5-HT and 2-Me-5-HT in the presence of TTX, can be explained in several ways: *a*) Part of the response to 5-HT might be mediated by a neurogenic but TTX-insensitive mechanism. Such a mechanism might be true if the 5-HT_3 receptors are present on the nerve terminals, or if action potentials are involved that utilize Ca^{2+} influx instead of Na^+ influx. The existence of TTX-resistant (Ca^{2+}-mediated) action potentials has been demonstrated in myenteric neurons

(Hirst & Spence, 1973; North, 1973). In preparations similar to ours, Costa & Furness (1979) observed that, in the presence of TTX (0.63 μM), hyoscine was still able to inhibit contractions to 5-HT. Hence, under these conditions, stimulation of 5-HT receptors still evoked the release of acetylcholine. This observation favours the possibility that these phenomena have a neuronal but TTX-insensitive nature. *b*) 5-HT_3 receptors might be located on non-neuronal tissue, i.e. directly on the smooth muscle membranes. To date, however, 5-HT_3 receptors have been found to be exclusively located on neurons (Richardson & Engel, 1986; Kilpatrick *et al.*, 1990). Therefore, this latter hypothesis does not seem very likely. *c*) The applied TTX concentration was insufficient to block neuronally mediated responses. This hypothesis must be rejected since it was demonstrated that the inhibitory effect against 5-HT and 5-MeOT of TTX 0.3 μM did not differ from the effects of TTX 1 μM or 3 μM. Hence, it is proposed that 5-HT_3 receptors are present in this preparation, which might be partially located on TTX-sensitive nerves and partially on nerve terminals or, possibly, on neurons that utilize TTX-insensitive mechanisms.

Ketanserin is known as a selective 5-HT_2 receptor antagonist (Van Nueten *et al.*, 1981; Leysen *et al.*, 1982). In the absence and presence of TTX, ketanserin inhibited the 5-HT-induced responses, suggesting that also 5-HT_2 receptors are involved in the contractions to 5-HT. The presence of 5-HT_2 receptors on the smooth muscle cells of the guinea-pig ileum has been demonstrated (Engel *et al.*, 1984). Thus, the most likely explanation for the observed inhibition of the 5-HT-induced contractions by ketanserin in the presence of TTX, is that, in the proximal colon of the guinea-pig, 5-HT_2 receptors are present on the smooth muscle. α-Me-5-HT is an agonist at 5-HT_2 receptors, though it is not very selective, as it has considerable activity at 5-HT_{1C} and 5-HT_4 receptors as well (Richardson & Hoyer, 1990; Bockaert *et al.*, 1992; Tadipatri & Saxena, 1992). The contractions to α-Me-5-HT were not significantly antagonized by ketanserin, which might be explained by a bias due to the considerable relaxations. In the presence of TTX, i.e. when the neuronally mediated relaxations are abolished, ketanserin did abolish the contractions to α-Me-5-HT, thus substantiating the proposal that muscular 5-HT_2 receptors are present in this preparation.

Ketanserin combined with granisetron or with tropisetron (0.3 μM), in the presence of TTX, was sufficient to eliminate responses to 5-HT. Since no response could be evoked by 5-HT under these conditions, 5-HT_2 and 5-HT_3 receptors appear to be the only types of 5-HT receptor responsible for the effects to 5-HT in the presence of TTX. An effect of 5-HT_2 or 5-HT_3 receptor antagonists against responses to 5-HT in the absence of TTX is to be expected, for it was proposed

above that muscular 5-HT_2 and 5-HT_3 receptors on TTX-insensitive neurons are present in this preparation. Indeed, ketanserin, granisetron and tropisetron (0.3 μM) inhibited 5-HT-induced responses, confirming these proposals.

Elswood and co-workers (Elswood *et al.*, 1991) demonstrated that 5-HT_4 receptors are present in the guinea-pig proximal colon. The results of our study support these findings. Blockade of the 5-HT_3 receptors with either granisetron or tropisetron 0.3 μM (equipotent at 5-HT_3 receptors [Butler *et al.*, 1990]), inhibited the contractions to 5-HT to an equal extent. When the preparations were incubated with tropisetron in a ten-fold higher concentration, i.e. a concentration of tropisetron that also blocks 5-HT_4 receptor mediated responses (pA_2 = 6.0-6.7 [Bockaert *et al.*, 1992]) the contractile responses were abolished, leaving only relaxations to occur. Thus the difference in the inhibition by tropisetron 0.3 μM and 3 μM must be due to the elimination of the 5-HT_4 receptor mediated contractions. This hypothesis is supported by a second set of observations. While the putative 5-HT_4 receptor was pharmacologically isolated through blockade of 5-HT_2 and 5-HT_3 receptors by a combination of ketanserin and granisetron, tropisetron 3 μM again potently inhibited the contractions to 5-HT. Since, in the presence of TTX, only 5-HT_2 and 5-HT_3 receptor-mediated responses could be demonstrated, it is proposed that only neuronal 5-HT_4 receptors are present.

5-MeOT is an agonist at some 5-HT_1 receptor subtypes and at 5-HT_2 and 5-HT_4 receptors, but lacks activity at 5-HT_3 receptors (Leff & Martin, 1988; Craig *et al.*, 1990; Bockaert *et al.*, 1992; Tadipatri & Saxena, 1992). In the presence of TTX, the contractions to 5-MeOT were abolished by ketanserin, confirming the presence of postjunctional 5-HT_2 receptors. In the absence of TTX, ketanserin apparently was not very effective in inhibiting the contractions to 5-MeOT. This could be caused, as with α-Me-5-HT (see above), by a bias of the contractile response by the concomitant relaxation. In the presence of TTX, 5-HT_4 receptor-mediated responses of 5-MeOT could not be shown, since the presence of tropisetron 3 μM did not result in enhanced inhibition as compared to TTX. However, in the absence of TTX, tropisetron 3 μM strongly inhibited the responses to 5-MeOT. Thus, also from the results with 5-MeOT, it can be concluded that the guinea-pig proximal colon is endowed with neuronal 5-HT_4 receptors.

With electrophysiological techniques, it has been shown that the enteric neurons of both the guinea-pig antrum and small intestine are endowed with inhibitory 5-HT_{1A} receptors and putative 5-HT_{1P} receptors (Mawe *et al.*, 1986; Tack *et al.*, 1992). It is thus quite possible that one or both of these receptors are also present on the enteric neurons of the guinea-pig proximal colon. However, no experiments with respect to these receptors were done in this study.

Conclusions: 5-HT induces contractions of the longitudinal muscle of the guinea-pig proximal colon, through the stimulation of 5-HT_2 receptors on the smooth muscle cells and 5-HT_3 receptors and putative neuronal 5-HT_4 receptors. 5-HT-evoked relaxations are mediated *via* an unknown neuronal receptor.

References

Bockaert J, Fozard, JR, Dumuis A, Clarke DE (1992) The 5-HT_4 receptor: a place in the sun. *Trends Pharmacol Sci* 13: 141-145.

Bradley PB, Engel G, Feniuk W, Fozard JR, Humphrey PPA, Middlemiss DN, Mylecharane EJ, Richardson BP, Saxena PR (1986) Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology* 25: 563-576.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1992) Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*. *Br J Pharmacol* 107: 756-761.

Brittain RT, Butler A, Coates IH, Fortune DH, Hagan R, Hille JM, Humber DC, Humphrey PPA, Ireland SJ (1987) GR 38032F, a novel selective 5-HT_3-receptor antagonist. *Br J Pharmacol* 90: 87P.

Butler A, Elswood CJ, Burridge J, Ireland SJ, Bunce KT, Kilpatrick GJ, Tyers MB (1990) The pharmacological characterization of 5-HT_3-receptors in three isolated preparations derived from guinea-pig tissues. *Br J Pharmacol* 101: 591-598.

Costa M, Furness JB (1979) The sites of action of 5-hydroxytryptamine in nerve-muscle preparations from the guinea-pig small intestine and colon. *Br J Pharmacol* 65: 237-248.

Craig DA, Eglen RM, Walsh LKM, Perkins LA, Whiting RL, Clarke DE (1990) 5-Methoxytryptamine and 2-methyl-5-hydroxytryptamine-induced desensitization as a discriminative tool for the 5-HT_3 and putative 5-HT_4 receptors in guinea-pig ileum. *Naunyn-Schmiedeberg's Arch Pharmacol* 342: 9-16.

Dumuis A, Sebben M, Cory R, Bockaert J (1988) A non-classical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol Pharmacol* 34: 880-887.

Elswood CJ, Bunce KT(1992) Investigation of the 5-HT receptor mediating relaxation in guinea-pig proximal colon. *J Pharm Pharmacol* 44: 264-266.

Elswood CJ, Bunce KT, Humphrey PPA(1991) Identification of putative 5-HT_4 receptors in guinea-pig ascending colon. *Eur J Pharmacol* 196: 149-155.

Engel G, Hoyer D, Kalkman HO, Wick MB (1984) Identification of 5-HT_2 receptors on longitudinal muscle of the guinea-pig ileum. *J Receptor Res* 4: 113-126.

Furness JB, Llewellyn-Smith IJ, Bornstein JC, Costa M (1988) Chemical neuroanatomy and the analysis of neuronal circuitry in the enteric nervous system. In: *Handbook of chemical neuroanatomy (Vol. 6: The peripheral nervous system)* (Björklund, Hökvelt, Owman Eds.) Elsevier, Amsterdam-New York-Oxford: pp.161-218.

Gershon MD (1967) Effects of tetrodotoxin in innervated smooth muscle preparations. *Br J Pharmacol Chemother* 29: 259-279.

Gershon MD, Erde SM (1981) The nervous system of the gut. *Gastroenterology* 80: 1571-1594.

Gershon MD, Mawe GM, Branchek TA (1989) 5-Hydroxytryptamine and enteric neurones. In: *The peripheral actions of 5-hydroxytryptamine* (Fozard JR Ed.) Oxford University Press, Oxford, UK: pp 247-264.

Hirst GDS, Spence I (1973) Calcium action potentials in mammalian peripheral neurones. *Nature New Biol* 243: 54-56.

Kilpatrick GJ, Bunce KT, Tyers MB (1990) 5-HT_3-receptors. *Med Res Rev* 10: 441-475.

Kojima S (1991) Characterization of 5-hydroxytryptamine-induced relaxations of guinea-pig proximal colon. *Arch Int Pharmacodyn* 313: 23-32.

Kojima S, Shimo Y (1986) The sites of action of 5-hydroxytryptamine in the longitudinal muscle of the guinea-pig proximal colon. *Asia Pacific J Pharmacol* 1: 111-116.

Leff P, Martin GR (1988) The classification of 5-hydroxytryptamine receptors. *Med Res Rev* 8: 187-202.

Legay C, Saffrey MJ, Burnstock G (1984) Coexsistence of immunoreactive substance P and serotonin in neurones of the gut. *Brain Res* 302: 379-382.

Leysen JE, Niemegeers CJE, Van Nueten JM, Laduron PM (1982) [^{3}H]-Ketanserin (R 41 468), a selective ^{3}H-ligand for serotonin$_2$ receptor binding sites. *Mol Pharmacol* 21: 301-314.

Mawe GM, Branchek AT, Gershon MD (1986) Peripheral neural serotonin receptors: identification and characterization with specific antagonists and agonists. *Proc Natl Acad Sci USA* 83: 9799-9803.

North RA (1973) The calcium-dependent slow after-hyperpolarization in myenteric plexus neurones with tetrodotoxin-resistant action potentials. *Br J Pharmacol* 49: 709-710.

Onori L, Saltarelli P, Marcoli M, Lecchini S, Frigo GM, Tonini M (1984) 5-Hydroxytryptamine desensitization fails to modify GABA-induced inhibitory responses in the guinea-pig colon. *J Pharm Pharmacol* 36: 406-408.

Richardson BP, Engel G (1986) The pharmacology and function of 5-HT_3-receptors. *Trends Neurosci* 9: 424-428.

Richardson BP, Hoyer D (1990) Selective agonists and antagonists at 5-hydroxytryptamine receptor subtypes. In: *Serotonin: from cell biology to pharmacology and therapeutics* (Paoletti R. *et al.* Eds.), Kluwer Academic Publishers, Dordrecht: pp.265-276.

Sanger GJ, King FD (1988) From metoclopramide to selective gut motility stimulants and 5-HT_3 receptor antagonists. *Drug Des Deliv* 3: 273-295.

Sanger GJ, Nelson DR (1989) Selective and functional 5-hydroxytryptamine$_3$ receptor antagonism by BRL 43694 (granisetron). *Eur J Pharmacol* 159: 113-124.

Tack JF, Janssens G, Vantrappen G, Wood JD (1992) Actions of 5-hydroxytryptamine on myenteric neurons in guinea-pig gastric antrum. *Am J Physiol* 263: G838-G846.

Tadipatri S, Saxena PR (1992) Is 2-methyl-5-HT a selective 5-HT_3 receptor agonist? *Br J Pharmacol* 105 (suppl): 34P.

Van Nueten JM, Janssen PAJ, Van Beek J, Xhonneux R, Verbeuren TJ, Vanhoutte PM (1981) Vascular effects of ketanserin (R 41 468), a novel antagonist of 5-HT_2 serotonergic receptors. *J Pharmacol Exp Ther* 218: 217-320.

Wallenstein S, Zucker CL, Fleiss JL (1980) Some statistical methods useful in circulation research. *Circ Res* 41: 1-9.

Chapter 3

Substance P-induced contractions of the guinea-pig proximal colon through stimulation of post-junctional tachykinin NK_1 receptors

M.R. Briejer, L.M.A. Akkermans, A.L. Meulemans,
R.A. Lefebvre and J.A.J. Schuurkes

slightly modified: *Eur J Pharmacol* (1993) 250: 181-183

Abstract. The effects of three tachykinin NK_1 receptor antagonists and a tachykinin NK_2 receptor antagonist against substance P-induced contractions of the guinea-pig proximal colon longitudinal muscle were investigated. Atropine, tetrodotoxin and phosphoramidon did not affect the concentration-response curve for substance P (pEC_{50} = 7.8). The tachykinin NK_1 receptor antagonist, 2*S*,3*S*-cis-CP 96345, competitively inhibited the contractions due to substance P (pA_2 = 8.5; constrained pA_2 = 8.9), but at higher concentrations (≥ 0.3 μM), 2*S*,3*S*-cis-CP 96345 also depressed the concentration-response curve for methacholine. The species-selective tachykinin NK_1 receptor antagonists, WIN 51708 and WIN 62577 (both 1 μM), and the tachykinin NK_2 receptor antagonist, SR 48968 (0.3 μM), had no effect. It is concluded that substance P induces contractions through the stimulation of tachykinin NK_1 receptors on the smooth muscle cells. In this preparation, tachykinin NK_2 receptors do not seem to be involved in the contractile action of substance P.

Introduction

The endogenous neuropeptide, substance P, a member of the neurokinin family, is considered to be a neurotransmitter in the enteric nervous system (see: Barthó & Holzer, 1985). It is most potent at tachykinin NK_1 receptors, which have been found on both neurons and smooth muscle, but it also has activity at smooth muscle tachykinin NK_2 receptors and tachykinin NK_3 receptors on the nerves (Barthó & Holzer, 1985; Maggi *et al.*, 1990; Yau *et al.*, 1992). In preparations of guinea-pig ileum, the longitudinal muscle is contracted by substance P through the stimulation of tachykinin NK_1 receptors. In the ileum, tachykinin NK_1 receptors are probably mostly located on the smooth muscle, though few of the tachykinin NK_1 receptors may be located on the nerves, inducing the release of acetylcholine (Maggi *et al.*, 1990). In the proximal colon circular muscle, tachykinin NK_1 and NK_2 receptors are present on the smooth muscle (Santicioli *et al.*, 1993), but the tachykinin receptor(s) that mediates the contraction in response to substance P on longitudinal muscle preparations has not been characterized yet. In order to do so, we investigated the effects of three potent and selective non-peptide tachykinin NK_1 receptor antagonists, CP 96345 (Snider *et al.*, 1991), WIN 51708 and WIN 62577 (Appell *et al.*, 1992), and a tachykinin NK_2 receptor antagonist, SR 48968 (Emonds-Alt *et al.*, 1992; Advenier *et al.*, 1992; Maggi *et al.*, 1993), against substance P-induced contractions of a guinea-pig proximal colon longitudinal muscle preparation.

Materials and methods

Dunkin Hartley guinea-pigs (400-600 g) were decapitated and the proximal colon was removed. Intact segments of about 2.5 cm were suspended under a 2g load in 20ml organ baths filled with De Jalon solution at 37 °C and gassed with 95 % O_2, 5 % CO_2 for isotonic measurement. After half an hour stabilization, the strips were challenged twice with methacholine (0.3 μM). In each preparation, a cumulative concentration-response curve was made for substance P in the absence, and consecutively, in the presence of an antagonist or vehicle control (contact time 15 min) (for details, see: Briejer *et al.*, 1992).

Data analysis

Contractions due to substance P were expressed as a percentage of the contractions with substance P in the absence of an antagonist for each preparation. Means and their standard error (S.E.M.) were calculated for graphic representation. pEC_{50} values (± 95 % confidence limits) were determined from the concentration-response curves of the individual strips with the aid of linear regression analysis of the values lying within a range of 20-80 % of the maximum response. The pA_2-value was calculated with Schild regression analysis. Effects of antagonists were evaluated by comparing EC_{50} values and maximum contractions to substance P in the absence and presence of antagonists, using the analysis of variance in combination with Dunnett's *t*-test; $P < 0.05$ was considered significant. The number of animals used is denoted by n.

Drugs

Substance P, tetrodotoxin (Serva, FRG), methacholine (Merck, FRG), phosphoramidon (Sigma, Belgium), atropine (Janssen Chimica, Belgium), SR 48968 ((S)-N-methyl N-[4-(4-acetylamino-4-phenyl-piperidino) 2-(3,4-dichlorophenyl)-butyl] benzamide) (Sanofi, France), 2S, 3S-cis-CP 96345 ((2S, 3S)-cis-2-(diphenylmethyl) N-[(2-methoxyphenyl)-methyl] 1-azabicyclo-[2.2.2.]-octan 3-amine) (Janssen Research Foundation, Belgium), and were dissolved in distilled water. WIN 62577 (17-β-hydroxy-17-α-ethynyl-Δ-4-androstano-[3,2-b]-pyrimido-[1,2-a]-benzimidazole) and WIN 51708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano-[3,2-b]-pyrimido-[1,2-a]-benzimidazole) (Research Biochemicals Inc., USA) were dissolved in dimethyl sulfoxide, which in its final bath concentration (1 % v/v) did not affect contractions due to methacholine or substance P.

Results

Substance P induced contractions from 0.1 nM onwards with a fast onset, yielding a sigmoidal concentration-response curve (Figure 3.1) (EC_{50} = 16 nM, Table 3.1). The concentration-response curve for substance P was not affected by the neuronal blocker, tetrodotoxin (0.3 μM), the muscarinic cholinoceptor antagonist, atropine (0.3 μM), or the neutral endopeptidase inhibitor, phosphoramidon (1 μM) (see Table 3.1). 2S,3S-cis-CP 96345 inhibited the contractions due to substance P in a surmountable fashion (Figure 3.1). Schild analysis yielded a straight line with a slope that was not statistically different from unity (0.8 ± 0.3, 95 % confidence limits; Figure 3.1) yielding a pA_2 value of 8.9 (± 0.5, 95 % confidence limits). When the slope was constrained to 1, a pA_2-value of 8.5 (± 0.1, 95 % confidence limits) was obtained. Up to 0.1 μM, 2S,3S-cis-CP 96345 did not affect the concentration-response curve for methacholine. However, at 0.3 μM, it depressed the concentration-response curve for methacholine (maximum contraction depressed by about 20 %; not shown, n = 4). Neither WIN 51708 and WIN 62577 (both 1 μM), nor their solvent, dimethyl sulfoxide, affected the concentration-response curves for either substance P (Table 3.1) or methacholine (n = 4; not shown), nor did SR 48968 (0.3 μM) (Table 3.1).

Table 3.1 pEC_{50}-values ± 95% confidence limits of the concentration-response curves for substance P in the absence and presence of drugs.

drug	pEC_{50}	95% confidence limits	n
controls (pooled)	7.80	7.97-7.68	20
phosphoramidon (1 μM)	7.99	8.28-7.82	8
tetrodotoxin (0.3 μM)	7.74	8.13-7.54	6
atropine (0.3 μM)	7.89	8.31-7.68	6
SR 48968 (0.3 μM)	7.98	8.24-7.81	6
WIN 51708 (1 μM)	7.78	8.10-7.60	6
WIN 62577 (1 μM)	7.81	8.16-7.62	6

None of the mean EC_{50}-values was significantly different from its concomitant control mean EC_{50}-value.

Discussion

Phosphoramidon, a potent inhibitor of neutral endopeptidase, EC.3.4.24.11 ($IC_{50} \approx 10$ nM; Turner *et al.*, 1985), had no effect on substance P-induced contractions, indicating that inactivation of substance P by neutral endopeptidase did not play an important role in our experiments. Neither tetrodotoxin nor atropine affected the contractions due to substance P, which suggests that, in our preparation, substance P acts solely through the stimulation of receptors on the smooth muscle.

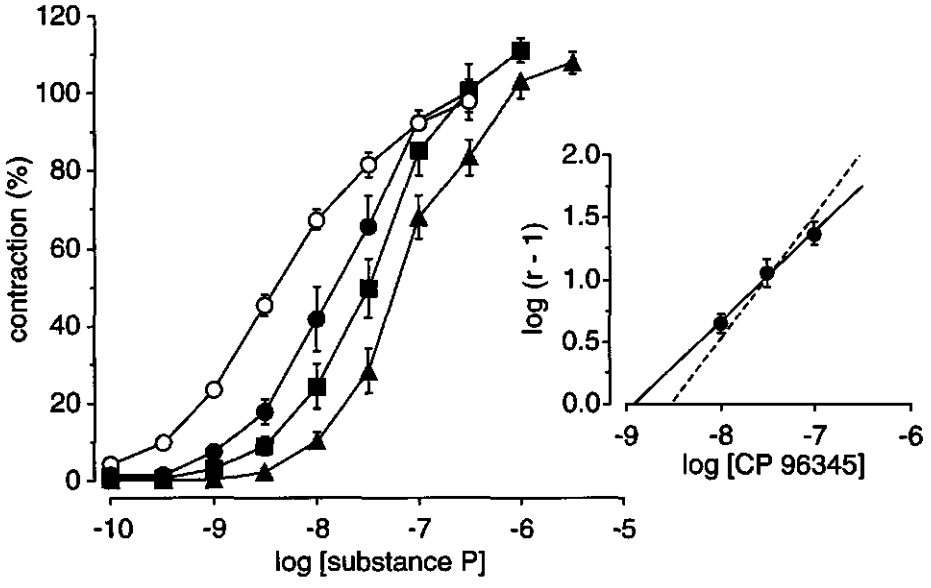


Figure 3.1 Effects of 2s,3s-cis-CP 96345 against contractions induced by substance P on the guinea-pig proximal colon longitudinal muscle: (O) control, or in the presence of 2s,3s-cis-CP 96345 1 nM (●), 30 nM (■), or 0.1 μM (▲). Mean values ± S.E.M. are shown; n = 6. The contractions were expressed as a percentage of the maximum contractions to substance P (0.3 μM) before 2s,3s-cis-CP 96345 was added. The inserted figure shows a Schild plot based on the data obtained with 2s,3s-cis-CP 96345: fitted line (solid), and fitted line with slope constrained to unity (dotted); *r* represents the concentration ratio.

The recent development of very potent and selective non-peptide tachykinin NK_1 and NK_2 receptor antagonists has led to the finding that there are species differences between these receptors. The selective tachykinin NK_1 receptor

antagonist, CP 96345 (Snider *et al.*, 1991), is about 100-fold more potent at guinea-pig type tachykinin NK_1 receptors than at rat type tachykinin NK_1 receptors (Sachais *et al.*, 1993; Barr & Watson, 1993). In our guinea-pig colon preparation, the substance P-induced contractions were competitively antagonized by 2S,3S-cis-CP 96345, yielding a pA_2 of 8.5, which is in good agreement with literature affinity values (pK_d = 8.3-8.4 at guinea-pig type tachykinin NK_1 receptors; Barr & Watson, 1993; Santicioli *et al.*, 1993). The tachykinin NK_1 receptor antagonists, WIN 51708 and WIN 62577, which bear no structural resemblance to CP 96345, have been reported to be much more potent at rat type tachykinin NK_1 receptors (K_i = 2.1-2.7 nM) than at guinea-pig type tachykinin NK_1 receptors (K_i > 10 μM) (Appell *et al.*, 1992). Hence, the observed lack of effect of both compounds at a concentration of 1 μM was to be expected and does not contradict the conclusion that tachykinin NK_1 receptors are involved in the contractile action of substance P. The selective tachykinin NK_2 receptor antagonist, SR 48968 (Emonds-Alt *et al.*, 1992; Maggi *et al.*, 1993), has been shown to be most potent at guinea-pig type tachykinin NK_2 receptors (pA_2 = 9.4-10.5) (Emonds-Alt *et al.*, 1992; Advenier *et al.*, 1992). SR 48968 (0.3 μM) did not affect contractile responses to substance P, which implies that tachykinin NK_2 receptors are not involved in substance P-induced contractions in this preparation.

It has been reported that CP 96345 has unspecific inhibitory activity on neurotransmission, probably due to its Ca^{2+} channel antagonist properties (Wang & Håkanson, 1992; Schmidt *et al.*, 1992). This might well explain the observed antagonism against contractions to methacholine at higher (0.3 μM) concentrations of CP 96345. These unspecific effects thus limit the use of CP 96345 to nanomolar concentrations.

In conclusion: substance P causes contractions of the guinea-pig proximal colon longitudinal muscle by stimulation of muscular tachykinin NK_1 receptors. Tachykinin NK_2 receptors do not seem to be involved in the contractile effect of substance P.

References

Advenier C, Rouissi N, Nguyen QT, Emonds-Alt X, Breliere J-C, Neliat G, Naline E, Regioli D (1992) Neurokinin A (NK_2) receptor revisited with SR 48968, a potent non-peptide antagonist. *Biochem Biophys Res Commun* 184: 1418-1424.

Appell KC, Fragale BJ, Loscig J, Singh S, Tomczuk BE (1992) Antagonists that demonstrate species differences in neurokinin-1 receptors. *Mol Pharmacol* 41: 772-778.

Barr AJ, Watson SP (1993) Non-peptide antagonists, CP-96,345 and RP 67580, distinguish species variants in tachykinin NK_1 receptors. *Br J Pharmacol* 180: 223-227.

Barthó L, Holzer P (1985) Search for a physiological role of substance P in the gastrointestinal motility. *Neuroscience* 16: 1-32.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1992) Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*. *Br J Pharmacol* 107: 756-761.

Emonds-Alt E, Vilain P, Goulaouic P, Proietto V, Van Broeck D, Advenier C, Naline E, Neliat G, Le Fur G, Brelière JC (1992) A potent and selective non-peptide antagonist of the neurokinin A (NK_2) receptor. *Life Sci* 50: PL101-PL106.

Maggi CA, Patacchini R, Giachetti A, Meli A (1990) Tachykinin receptors in the circular muscle of the guinea-pig ileum. *Br J Pharmacol* 101: 996-1000.

Maggi CA, Patacchini R, Giuliani S, Giachetti A (1993) *In vivo* and *in vitro* pharmacology of SR 48968, a non-peptide tachykinin NK_2 receptor antagonist. *Eur J Pharmacol* 234: 83-90.

Sachais BS, Snider RM, Lowe JA, Krause JE (1993) Molecular basis for the species selectivity of the substance P antagonist CP-96,345. *J Biol Chem* 268: 2319-2323.

Santicioli P, Giuliani S, Bartho L, Maggi CA (1993) Tachykinin NK-1 and NK-2 receptors in the circular muscle of the guinea-pig proximal colon. *Regul Pept* 46: 386-388.

Schmidt AW, McLean S, Heym J (1992) The substance P receptor antagonist CP-96,345 interacts with Ca^{2+} channels. *Eur J Pharmacol* 219: 491-492.

Snider MR, Constantine JW, Lowe III JA, Longo KP, Lebel WS, Woody HA, Drozda SE, Desai MC, Vinick FJ, Spencer RW, Hess HJ (1991) A potent nonpeptide antagonist of the substance P (NK1) receptor. *Science* 251: 435-437.

Turner AJ, Matsas R, Kenny AJ (1985) Are there neuropeptide-specific peptidases? *Biochem Pharmacol* 34: 1347-1356.

Wang Z-Y, Håkanson R (1992) (±)-CP-96,345, a selective tachykinin NK_1 receptor antagonist, has non-specific actions on neurotransmission. *Br J Pharmacol* 107: 762-765.

Yau WM, Mandel KG, Dorsett JA, Youther ML (1992) Neurokinin$_3$ receptor regulation of acetylcholine release from myenteric plexus. *Am J Physiol* 263: G659-G664.

Chapter 4

Cisapride and a structural analogue, R 76 186, are 5-hydroxytryptamine$_4$ (5-HT$_4$) receptor agonists on the guinea-pig colon ascendens

M.R. Briejer, L.M.A. Akkermans, A.L. Meulemans,
R.A. Lefebvre and J.A.J. Schuurkes

slightly modified: *Naunyn-Schmiedeberg's Arch Pharmacol* (1993) 347: 464-470

Abstract. The purpose of this study was to investigate whether the effects of cisapride and its close structural analogue R 76 186 on the isolated guinea-pig colon ascendens, are mediated through 5-HT_4 receptors.

Both cisapride and R 76 186 induced contractions in a concentration-dependent fashion, giving monophasic concentration-response curves (cisapride: EC_{50} = 0.12 μM, maximum effect = 40.3 % of methacholine-induced (0.3 μM) contractions; R 76 186: EC_{50} = 24 nM, maximum effect = 52.1 %). Blockade of either 5-HT_2 or 5-HT_3 receptors did not affect the responses to cisapride. However, tropisetron (in 5-HT_4 receptor blocking concentrations), and DAU 6285 and SDZ 205-557, two novel selective 5-HT_4 receptor antagonists, depressed the concentration-response curve to cisapride (to about 50 %), and the curve to R 76 186 was shifted to the right. The estimated pA_2 values were 6.6 (tropisetron), 6.3 (DAU 6285), and 7.5 (SDZ 205-557). However, none of these antagonisms was purely competitive as higher concentrations of these antagonists depressed the curve to R 76 186. Desensitization of the 5-HT_4 receptor with 5-methoxytryptamine (5-MeOT) inhibited the responses to cisapride, and abolished those to R 76 186. The contractions to cisapride and R 76 186 were sensitive to mutual antagonism, depressing their concentration-response curves.

Conclusions: Both cisapride and R 76 186 mediate their contractile effects in the guinea-pig colon ascendens through agonism at the 5-HT_4 receptor, though cisapride also uses a non-5-HT mechanism. R 76 186 is a selective and potent 5-HT_4 receptor agonist.

Introduction

Cisapride is a 4-amino-5-chloro-2-methoxy-substituted benzamide that is clinically effective in the treatment of gastrointestinal motility disorders such as non-ulcer dyspepsia, gastro-oesophageal reflux, and constipation (Reyntjens *et al.*, 1986; Müller-Lissner, 1985; 1987). *In vivo*, cisapride has been shown to stimulate motility in different animal species, and in gastrointestinal smooth muscle preparations of both animal and man, cisapride enhances contractility (Schuurkes, 1992). Its mechanism of action is however still unclear. *In vitro*, cisapride has been shown to interact with several 5-hydroxytryptamine (5-HT) receptors. It is an antagonist at 5-HT_{1A}, 5-HT_2 and 5-HT_3 receptors (Moriarty *et al.*, 1987; Van Nueten & Schuurkes, 1989; Leysen, 1990; Taniyama *et al.*, 1991). It has been hypothetised that its prokinetic action is related with agonistic activity at the recently discovered 5-HT_4 receptor (see for review: Bockaert *et al.*, 1992). The enhanced cholinergic transmission which is induced by stimulation of this 5-HT_4 receptor (Taniyama *et al.*, 1991; Kilbinger and Wolf, 1992), is proposed to be responsible for the prokinetic effects of cisapride and structurally related benzamides, such as renzapride, zacopride and metoclopramide (Tonini *et al.*, 1989; 1991; Bockaert *et al.*, 1992).

In the guinea-pig proximal colon, cisapride induces tonic contractions (Schuurkes *et al.*, 1985; Elswood *et al.*, 1991). The contractile effects in this prepa-

ration to renzapride and zacopride, two prokinetic benzamides that are structural analogues of cisapride, are sensitive to 5-HT_4 receptor blocking concentrations of tropisetron (ICS 205-930) (Elswood *et al.*, 1991). Besides a potent 5-HT_3 receptor antagonist, tropisetron is a low potency 5-HT_4 receptor antagonist (pA_2 = 6.0 - 6.7, Bockaert *et al.*, 1992). Recently, two novel, more potent and more selective 5-HT_4 receptor antagonists have been introduced, i.e. SDZ 205-557 (Buchheit *et al.*, 1991; 1992) and DAU 6285 (Turconi *et al.*, 1991; Dumuis *et al*, 1992; Schiavone *et al.*, 1992). A second manner to investigate 5-HT_4 receptor mediated responses, is the selective desensitization of this receptor with 5-methoxytryptamine (5-MeOT), a 5-hydroxytryptamine analogue that lacks activity at 5-HT_3 receptors (Fozard, 1985; Craig *et al.*, 1990; Meulemans & Schuurkes, 1992).

The aim of our study was to investigate whether cisapride is a 5-HT_4 receptor agonist on the guinea-pig colon ascendens, utilizing both the established pharmacological tools (i.e. receptor desensitization with 5-MeOT and antagonism with tropisetron) and the two novel antagonists (i.e. SDZ 205-557 and DAU 6285). As a benzamide comparator, a structural analogue to cisapride, R 76 186, was also incorporated into this study. R 76 186 is the most potent and a highly efficacious 5-HT_4 receptor agonist in the mouse colliculi neurons (Bockaert *et al.*, 1991).

Materials and methods

Dunkin-Hartley guinea-pigs of both sexes, weighing 400-600 g, were killed by cervical dislocation. The ascending colon was removed, and the luminal contents were washed out with De Jalon's solution (mM: KCl 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, NaCl 155, glucose 2.8). The mesentery was carefully removed. Starting at the proximal end, about 1 cm distal from the caecum, the colon was divided into four segments of about 2.5 cm. These intact segments were individually mounted vertically for isotonic measurement into a tissue bath containing 20 ml De Jalon's solution. This solution was kept at 37 °C and gassed with 95% O_2, 5 % CO_2. The strips were subjected to a preload of 2 g and allowed to stabilize for 20 min. After stabilization, the response of the longitudinal muscle to 0.3 μM methacholine (about 60 % of maximum contraction) was measured. The measurement was repeated after washing (i.e. replacing the bathing fluid twice) and stabilization (10 min) until the response to methacholine remained constant (usually 3 times).

Protocols

Strips were incubated with the 5-MeOT (desensitization of 5-HT_4 receptors), R 76 186, cisapride (for testing mutual antagonism effects), a 5-HT receptor antagonist, or solvent for at least 15 min. Subsequently, one concentration of cisapride or R 76 186 was added to the bath. The response was monitored for 5 min at most. Tropisetron had an intrinsic effect (ranging from about 5 % (of methacholine-induced contractions, see below) at 1 μM to about 10 % at 10 μM), but the strips always had returned to baseline length before the agonist was administered. The applied 5-HT_2 and 5-HT_3 receptor antagonists, as well as DAU 6285 and SDZ 205-557, were without intrinsic effects. The concentration-response curves of cisapride and R 76 186 either in the absence or presence of an antagonist were obtained by single dose administration (one concentration per preparation). The fact that repeated benzamide administration results in reduced contractions necessitated these single point measurements. A disadvantage of these single point measurements is that biological variability gives rise to more fluctuation than in the case of multipoint measurements. All responses were expressed as a percentage of the mean of the last two contractions induced by methacholine 0.3 μM.

To investigate the specificity of the applied antagonists and 5-MeOT, they were tested against methacholine-induced contractions. Methacholine was applied cumulatively (0.5 log concentration intervals) to establish concentration-response curves. First, a concentration-response curve to methacholine in the absence of antagonist was established, and subsequently, after 30 min of stabilization and repeated washing, a second concentration-response curve was established in the presence of either solvent or antagonist. The incubation period was at least 15 min in this case as well. The responses of the second curves were expressed relatively to the maximum effect to methacholine obtained in the first curve, occurring at 10 μM.

Data analysis

For graphical representation means ± standard error of the means were calculated. Mean values were compared with the Scheffé *F*-test or the Dunnett *t*-test, when appropriate, for multiple comparisons. The level of $P < 0.05$ was considered to indicate significant difference. EC_{50} values and a range of the EC_{50} values ± their standard errors were determined with the aid of the computer software SAS (version 6, SAS Institute Inc., North Carolina), performing an iterative non-linear curve-fit according to the method of Marquardt. The pA_2 values were estimated with the Schild-equation:

$$pA_2 = {}^{10}\log (r - 1) - {}^{10}\log \text{(molar concentration of antagonist)}$$

in which r represents the concentration ratio.

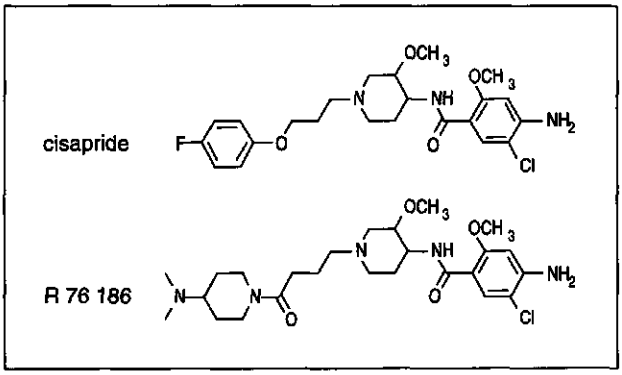


Figure 4.1 Structural formulas of the 4-amino-5-chloro-2-methoxy-substituted benzamides cisapride and R 76 186.

Drugs

Solutions of R 76 186 (cis-4-amino-5-chloro-N-[1-[4-[4-(dimethylamino)-1-piperidinyl]-4-oxo-butyl]-3-methoxy-4-piperidinyl] 2-methoxybenzamide) and cisapride (cis-4-amino-5-chloro-N-[1-[3-(4-fluorophenoxy) propyl]-3-methoxy-4-piperidinyl] 2-methoxybenzamide monohydrate), (Figure 4.1), DAU 6285 HCl (endo-6-methoxy-8-methyl-8-azabicyclo-[3.2.1]-oct-3-yl 2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxylate hydrochloride), ketanserin tartrate ((+)-3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]-ethyl]-2,4-(1H,3H)-quinazoline-di-one monotartrate), SDZ 205-557 (2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethyl-amino) ethyl ester), pirenperone (3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]-ethyl]-2-methyl-4H-pyrido-[1,2-a]-pyrimidin-4-one) (Janssen Research Foundation, Belgium), tropisetron HCl ((8-methyl-8-azabicyclo-[3.2.1]-oct-3yl) 1H-indole 3-carboxylate monohydrochloride) (ICS 205-930; Sandoz, Switzerland), granisetron HCl (endo-1-methyl-N-(9-methyl-9-azabicyclo-[3.3.1]-non-3-yl) 1H-indazole-3-carboxamide monohydrochloride) (BRL 43694; SmithKline Beecham, United Kingdom), ondansetron (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)-methyl] 4H-carbazol-4-one) (GR 38032F; Glaxo, United Kingdom), 5-methoxytryptamine HCl, (Janssen Chimica, Belgium), methacholine HCl (E. Merck, Germany) were added to the

organ bath solution in volumes ≤ 1 % of the bath volume. DAU 6285 was a kind gift from dr. C.A. Rizzi, Boehringer Ingelheim, Italy. Methacholine, tropisetron, ondansetron, granisetron, DAU 6285, ketanserin and 5-methoxytryptamine were dissolved in distilled water. Cisapride, pirenperone, SDZ 205-557 and R 76 186 were dissolved in distilled water acidified with tartaric acid in the stock solution (pH ≥ 3 in the stock solution). The solvent had no effect on the resting length of the preparation and on the methacholine-induced contractions.

Results

Cisapride concentration-dependently induced contractions from 10 nM onwards. The contractions had a rather slow onset, reaching a maximum after 2-4 min, and usually had a tonic character. The concentration-response curves to cisapride appeared monophasic (Figure 4.2, *left panel*) with an EC_{50} of 0.12 (0.09-0.15) μM and a maximum effect at 1 μM of 40.3 ± 1.9 % (n = 8-35).

Table 4.1 Estimated pA_2 values of 5-HT_4 receptor antagonists against R 76 186 in the guinea-pig colon ascendens in comparison to literature pA_2 values.

	estimated pA_2	literature pA_2
tropisetron	6.6	6.0-6.7[a]
DAU 6285	6.3	6.4-7.4[b]
SDZ 205-557	7.5	7.2-7.7[c]

[a]Bockaert *et al.*, 1992; [b]Turconi *et al.*, 1991; Schiavone *et al.*, 1992; Dumuis *et al.*, 1992; [c]Buchheit *et al.*, 1992.

Neither the 5-HT_2 receptor antagonists ketanserin (30 nM) and pirenperone (30 nM), nor the 5-HT_3 receptor antagonists ondansetron (1 μM), granisetron (0.1 μM) and tropisetron (0.1 μM), significantly affected the contractile response to cisapride (0.3 μM) (n = 6 - 14, results not shown).

R 76 186 induced contractions from 1 nM onwards in a concentration-dependent fashion. The contractions had a rather fast onset, reaching the maximum effect usually within 1 min, and were short-lasting. The concentration-response curve to R 76 186 appeared monophasic (Figure 4.2, *middle panel*) with an EC_{50} of 24 (21-27) nM and a maximum effect at 0.1 μM of 52.1 ± 3.0 % (n = 6 - 31).

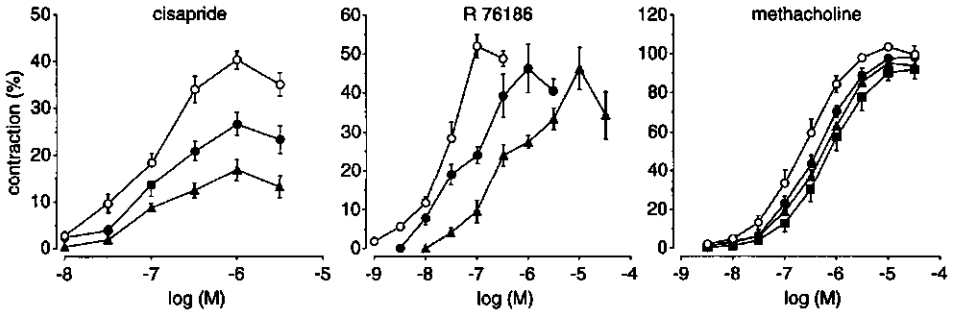


Figure 4.2 Effects of tropisetron on the concentration-response curves to cisapride (*left panel*), R 76 186 (*middle panel*) and methacholine (*right panel*). Shown are the control curves (O) and the curves in the presence of tropisetron 1μM (●), 3 μM (▲), and 1 μM (■). The contractile responses to cisapride and R 76 186 were expressed as a percentage of contractions to methacholine 0.3 μM. However, the contractile responses to methacholine were expressed as a percentage of the maximum effect to methacholine (at 10 μM). Means ± s.e.m., n = 4 - 35.

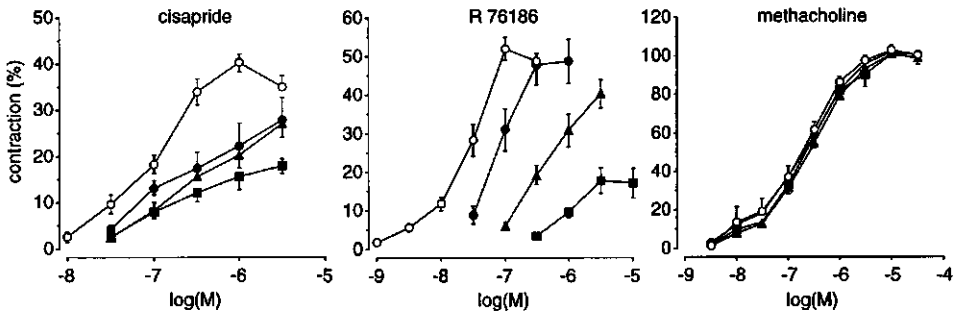


Figure 4.3 Effects of DAU 6285 on the concentration-response curves to cisapride (*left panel*), R 76 186 (*middle panel*) and methacholine (*right panel*). Shown are the control curves (O) and the curves in the presence of DAU 6285 1 μM (●), 3 μM (▲), and 10 μM (■). The contractile responses to cisapride and R 76 186 were expressed as a percentage of contractions to methacholine 0.3 μM. However, the contractile responses to methacholine were expressed as a percentage of the maximum effect to methacholine (at 10 μM). Means ± s.e.m., n = 6 - 35.

Tropisetron

Tropisetron (1 μM and 3 μM) significantly inhibited the contractions to cisapride (n = 6 - 12) (Figure 4.2, *left panel*) and R 76 186 (n = 5 - 20) (Figure 4.2, *middle panel*), but in a different way. The responses evoked by cisapride were inhibited in a non-surmountable way (Figure 4.2, *left panel*). The concentration-

response curve to R 76 186 however was shifted to the right without depression of the maximum effect. Schild analysis yielded a slope of 2.2. Though this value definitely exceeds unity, significance could not be tested, since only two concentrations of tropisetron could be applied. The estimated pA_2 (determined with the Schild-equation using only tropisetron 1 μM) yielded a value of 6.6 (Table 4.1). The inhibition by tropisetron of the responses to cisapride and R 76 186 was not fully specific, since at 3 μM, it tended to inhibit the responses to methacholine (a significant depression of the methacholine concentration-response curve was reached using tropisetron 10 μM) (Figure 4.2, *right panel*) though to a much lesser extent (n = 4). Because of this non-specific inhibitory action, tropisetron was not tested against cisapride and R 76 186 in concentrations exceeding 3 μM.

DAU 6285

DAU 6285 significantly inhibited the contractions to cisapride in a non-surmountable fashion, the inhibition by DAU 6285 10 μM (n = 6) being not different from the inhibition by DAU 6285 1 μM (n = 6 - 8) and 3 μM (n = 6) (Figure 4.3, *left panel*). DAU 6285 also significantly inhibited the contractions to R 76 186. The inhibition by DAU 6285 1 μM (n = 8) was surmountable (maximum effect to R 76 186 not significantly different from control), but higher concentrations of DAU 6285 (3 μM, n = 6; 10 μM, n = 6) significantly depressed the maximum effect to R 76 186 (Figure 4.3, *middle panel*). The estimated pA_2, calculated only with the concentration-response curve to R 76 186 in the presence of DAU 6285 1 μM, was 6.3 (Table 4.1). The inhibition by DAU 6285 of the effects to cisapride and R 76 186 appeared specific, for the concentration-response curve to methacholine was not affected by DAU 6285 up to 10 μM (n = 6) (Figure 4.3, *right panel*).

SDZ 205-557

SDZ 205-557 inhibited the effects of cisapride (n = 6 - 10), again in a non-surmountable fashion (Figure 4.4, *left panel*). SDZ 205-557 significantly inhibited the contractile effects to R 76 186 at a concentration as low as 0.1 μM SDZ 205-557 (n = 6 - 8) (Figure 4.4, *middle panel*). At 0.1 μM, the antagonism by SDZ 205-557 seemed surmountable (maximum effect to R 76 186 not significantly different from control; estimated pA_2 = 7.5, Table 4.1). Up to 10 μM, SDZ 205-557 did not affect the concentration-response curve to methacholine (n = 4) (Figure 4.4, *right panel*).

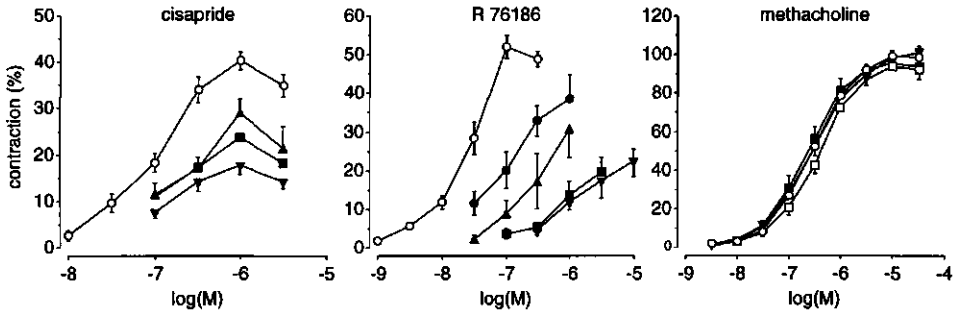


Figure 4.4 Effects of SDZ 205-557 on the concentration-response curves to cisapride (*left panel*), R 76 186 (*middle panel*) and methacholine (*right panel*). Shown are the control curves (O) and the curves in the presence of SDZ 205-557 0.1 μM (●), 0.3 μM (▲), 1 μM (■), 3 μM (▼), and 10 μM (□). The contractile responses to cisapride and R 76 186 were expressed as a percentage of contractions to methacholine 0.3 μM. However, the contractile responses to methacholine were expressed as a percentage of the maximum effect to methacholine (at 10 μM). Means ±-s.e.m., n = 4 - 35.

5-MeOT

5-MeOT (3 μM: a concentration causing maximum contractions [Briejer *et al.*, 1993]) induced a quick initial relaxation, followed by a much larger contraction (47.2 ± 2.8 %), and subsequently a more sustained relaxation. The effects of 5-MeOT had disappeared after 15 min, so by the time the second agonist (cisapride, R 76 186 or methacholine) was administered, the strips had returned to resting length. Preincubation of the strips with 5-MeOT (3 μM) resulted in significantly decreased contractions to cisapride as compared to control (n = 7-8) (Figure 4.5, *left panel*). The responses to R 76 186 were abolished by desensitization with 5-MeOT (3 μM; n = 4) (Figure 4.5, *middle panel*). The specificity for 5-HT receptors is illustrated by the fact that the concentration-response curve to methacholine was not affected by preincubation with 5-MeOT (3 μM; n = 4) (Figure 4.5, *right panel*).

Mutual antagonism

The responses to cisapride were sensitive to antagonism by R 76 186 0.3 μM (n = 6) (Figure 4.6, *left panel*). The R 76 186 evoked responses were also sensitive to antagonism with cisapride 0.3 μM (n = 6) (Figure 4.6, *right panel*).

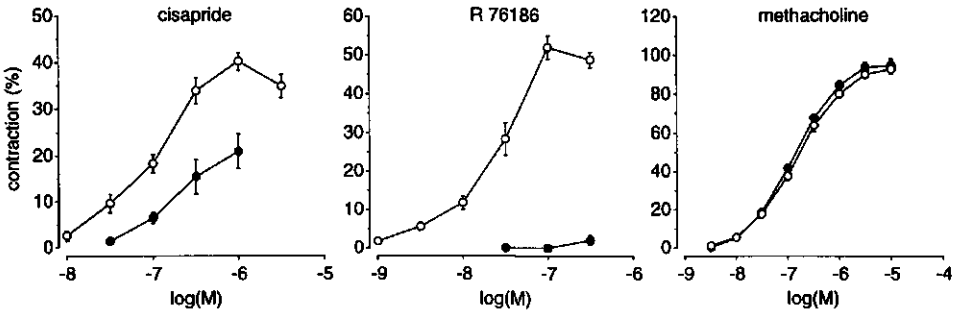


Figure 4.5 Effects of 5-MeOT on the concentration-response curves to cisapride (*left panel*), R 76 186 (*middle panel*) and methacholine (*right panel*). Shown are the control curves (O) and the curves in the presence of 5-MeOT 3 μM (●). The contractile responses to cisapride and R 76 186 were expressed as a percentage of contractions to methacholine 0.3 μM. However, the contractile responses to methacholine were expressed as a percentage of the maximum effect to methacholine (at 10 μM). Means ± s.e.m., n = 4 - 8.

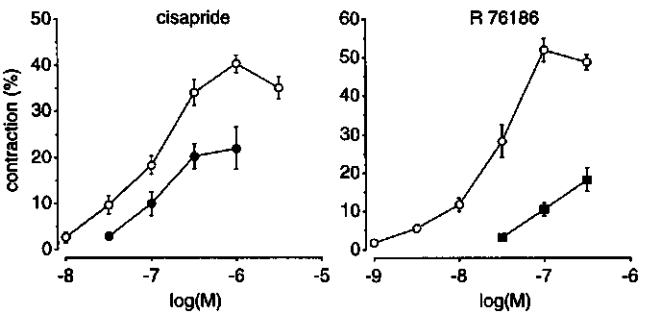


Figure 4.6 Effects of mutual antagonism on the concentration-response curves to cisapride (*left panel*) and R 76 186 (*right panel*). Shown are the control curves (O) and the curves in the presence of 0.3 μM R 76 186 (*left panel*, ●), and cisapride 0.3 μM (*right panel*, ■). The contractile responses to cisapride and R 76 186 were expressed as a percentage of contractions to methacholine 0.3 μM. Means ± s.e.m., n = 6.

Discussion

Our study clearly demonstrates that 5-HT_4 receptors are involved in the contractile action of cisapride on the guinea-pig ascending colon. The lack of effect by the selective 5-HT_2 receptor antagonists ketanserin and pirenperone

(Leysen, 1990), and by the selective 5-HT_3 receptor antagonists ondansetron, granisetron and the non-selective tropisetron (nanomolar concentrations) (Butler *et al.*, 1990; Kilpatrick *et al.*, 1990), is in good agreement with existing data that cisapride interacts with these receptor subtypes without intrinsic effect (Moriarty *et al.*, 1987; Van Nueten and Schuurkes, 1989; Leysen, 1990). The 5-HT_4 receptor antagonists tropisetron (micromolar concentrations), DAU 6285 and SDZ 205-557 (Buchheit *et al.*, 1991; 1992; Turconi *et al.*, 1991; Dumuis *et al*, 1992; Schiavone *et al.*, 1992) all inhibited the responses evoked by cisapride, thus showing that at least part of the response to cisapride is mediated through 5-HT_4 receptors. Desensitization of the 5-HT_4 receptor with 5-MeOT (Fozard, 1985; Craig *et al.*, 1990; Meulemans & Schuurkes, 1992) inhibited the effects to cisapride, thus giving extra evidence that this receptor subtype is involved.

Stimulation of the 5-HT_4 receptor, at least in the guinea-pig ileum and probably also in the colon, induces facilitation of the cholinergic transmission (Schuurkes *et al.*, 1985; Taniyama *et al.*, 1991; Kilbinger & Wolf, 1992). Hence, it is important to ascertain that a 5-HT_4 receptor antagonist does not antagonise cholinergic responses. The concentration-response curve to methacholine, an agonist at the muscarinic acetylcholine receptor, was not affected by preincubation of the strips with either DAU 6285, SDZ 205-557 or 5-MeOT, indicating that the inhibitory effects against cisapride of these agents were specific. Tropisetron (10 μM) however did affect the methacholine-induced contractions. Baxter *et al.* (1991) reported competitive antagonism at the muscarinic acetylcholine receptor (pA_2 = 5.4) by tropisetron in rat oesophageal tunica muscularis. In our preparation, however, the maximum effect of methacholine was significantly depressed by tropisetron 10 μM; tropisetron thus did not seem to antagonize the contractions to methacholine in a competitive fashion. Scholtysik *et al.* (1988) showed that tropisetron blocks cardiac K^+, Na^+ and Ca^{2+}-channels. Hence, tropisetron may inhibit the methacholine-induced responses by affecting the membrane potential of the smooth muscle cells in our preparation. Still, the inhibition by tropisetron of the methacholine-induced responses was much less pronounced than the inhibition against cisapride-induced contractions, thus providing extra evidence for the 5-HT_4 receptor agonist component in the contractile effect of cisapride.

Blockade with 5-HT_4 receptor antagonists and desensitization with 5-MeOT all seem to reveal a rest response to cisapride which is not mediated by 5-HT_4 receptors. Stimulation of 5-HT_1 receptors in this tissue induces relaxations rather than contractions (Kojima, 1991; Elswood and Bunce, 1992; Briejer *et al.*, 1992). The relaxations induced by 5-MeOT are caused by stimulation of these 5-HT_1

receptor (Briejer *et al.*, 1992; 1993). Since blockade of 5-HT_2 and 5-HT_3 receptors had no effect on cisapride induced contractions, it seems likely that the second component in the contractile action of cisapride is mediated by a non-5-HT receptor. The contractions to the 5-HT_4 receptor agonist 5-MeOT were fast and short-lasting, whereas, in contrast, those to cisapride were developing more slowly and had a more tonic character. This may also indicate that cisapride does not act solely through a 5-HT_4 receptor mechanism. Sanger (1987) suggested that metoclopramide (the first clinically applied gastrointestinal prokinetic benzamide) increases electrical field stimulation-evoked contractions of guinea-pig ileum preparations by at least two different mechanisms, only one of which involves activation of enteric 5-HT-like (i.e. 5-HT_4) receptors. In isolated guinea-pig gastric smooth muscle cells, it was suggested that cisapride acts as an agonist at muscarinic acetylcholine receptors (Hasler *et al*, 1991). Schuurkes & Van Nueten (1989) showed that, in a preparation identical to ours, the TTX-resistant component in the contraction to cisapride was no more sensitive to atropine. This suggests that cisapride does not act at (myogenic) muscarinic acetylcholine receptors in the guinea-pig colon. Clearly, the non-5-HT component in the action of cisapride deserves further investigation.

Table 4.2. Comparison of EC_{50} values and efficacies of cisapride and R 76 186, obtained in the guinea-pig colon ascendens and in mouse colliculi neurons (Bockaert *et al.*, 1991).

	EC_{50}	efficacy
colliculi neurons		
cisapride	72 nM	142 %
R 76186	27 nM	134 %
ratio	2.7	1.1
proximal colon		
cisapride	115 nM	40.3 %
R 76186	24 nM	52.1 %
ratio	4.8	0.8

In our experiments, R 76 186, a substituted benzamide that is structurally related to cisapride, appeared to be an agonist at the 5-HT_4 receptor, since it was inhibited by the applied 5-HT_4 receptor antagonists (with estimated pA_2 values that are in good agreement with values from the literature, see Table 4.1) as well as by receptor desensitization with 5-MeOT. In fact, the latter procedure abolished

the contractions to R 76 186, indicating that R 76 186, at least up to 0.3 μM, is a selective 5-HT_4 receptor agonist. This view is supported by the fact that DAU 6285 and SDZ 205-557, which are selective 5-HT_4 receptor antagonists, also completely inhibited the contractions to R 76 186 up to 0.3 μM. In primary cultures of foetal mouse colliculi neurons, the system in which 5-HT_4 receptors were first identified by Dumuis and colleagues (1988), R 76 186 was a very potent agonist at 5-HT_4 receptors with a high efficacy (Table 4.2) (Bockaert *et al.*, 1991). In our experiments, R 76 186 was also very effective at the 5-HT_4 receptor with a high affinity. Both the potency and efficacy ratios of the results of Bockaerts study (collicular neurons) and ours correspond well (Table 4.2). This reflects the similarity of action of cisapride and R 76 186 in these tissues. The 5-HT_4 receptor is subjected to rapid desensitization (Craig *et al.*, 1990; Meulemans & Schuurkes, 1992). Since cisapride and R 76 186 mutually antagonized their responses, both compounds must have at least one site of action in common, supposedly the 5-HT_4 receptor site.

The elucidation of the site of action of the benzamides is endowed with a number of peculiar observations, to which our study with cisapride and R 76 186 also adds some. The determination of the kind of antagonism by the applied 5-HT_4 receptor antagonists against cisapride is troubled by the second, non-5-HT component in the action of cisapride (see above). From the antagonism against R 76 186, it is clear, that none of the antagonists inhibited contractions to R 76 186 in a competitive fashion, though the inhibition by tropisetron was surmountable. In most preparations, tropisetron (in micromolar concentrations) is a competitive 5-HT_4 receptor antagonist (Bockaert *et al.*, 1992), but in the guinea-pig colon it antagonised the contractions to 5-HT and 5-MeOT, and also to the benzamides zacopride and renzapride, in a non-surmountable fashion (Elswood *et al.*, 1991). In the guinea-pig ileum, SDZ 205-557 antagonised responses to 5-HT and 5-MeOT competitively, but not those to renzapride (Buchheit *et al.*, 1991; 1992). DAU 6285 is reported to inhibit both responses to 5-HT and 5-MeOT, as well as to cisapride in a competitive fashion (Schiavone *et al*; 1992; Turconi *et al.*, 1991; Tonini *et al.*, 1992). In the mouse colliculi neuron system, R 76 186 and tropisetron could displace [^{3}H]-renzapride binding, whereas 5-HT and 5-MeOT could not (Bockaert *et al.*, 1991). They hypothetized that the 5-HT_4 receptor might contain two different subsites, one for the benzamides, the other for 5-HT and indoles. In this model, binding of 5-HT or benzamides at their specific subsites would be mutually exclusive (Bockaert *et al.*, 1991). In guinea-pig myenteric neurons, a binding site for the benzamides S-zacopride (but not its enantiomer R-zacopride) and renzapride was demonstrated, distinct from the binding site for

5-HT. It was hypothetized that this benzamide site has a modulatory function in 5-HT transmission (Wade *et al.*, 1991). From all these observations, it is clear, that the interaction of the applied antagonists, the substituted benzamides and the 5-HT_4 receptor site, is complex and needs further study.

Conclusions: Cisapride and its analogue R 76 186 are agonists at the 5-HT_4 receptor in the guinea-pig colon ascendens. Cisapride appears to induce contractions not only through stimulation of the 5-HT_4 receptor, but also through a second mechanism, probably not involving 5-HT receptors. R 76 186, in contrast, appears to be a selective 5-HT_4 receptor agonist.

References

Baxter GS, Craig DA, Clarke DE (1991) 5-Hydroxytryptamine$_4$ receptors mediate relaxation of the rat oesophageal tunica muscularis mucosae. *Naunyn-Schmiedeberg's Arch Pharmacol* 343: 439-446.

Bockaert J, Fagni, L, Sebben M, Dumuis A (1991) Pharmacological characterization of brain 5-HT_4 receptors: relationship between the effects of indole, benzamide and azabicycloalkyl-benzimidazolone derivatives. In: *Serotonin: molecular biology, receptors and functional effects* (Fozard JR, Saxena PR Eds.), Birkhäuser Verlag, Switzerland, pp. 220-231.

Bockaert J, Fozard JR, Dumuis A, Clarke DE (1992) The 5-HT_4 receptor: a place in the sun. *Trends Pharmacol Sci* 13: 141-145.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1992) Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*. *Br J Pharmacol* 107: 756-761.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1993) Pharmacological characterization of 5-hydroxytryptamine receptor types in the guinea-pig proximal colon. *J Gastrointest Motil* 5: 239-246.

Buchheit K-H, Gamse R, Pfannkuche H-J (1991) SDZ 205-557, a selective antagonist at 5-HT_4 receptors in the isolated guinea-pig ileum. *Eur J Pharmacol* 200: 373-374.

Buchheit K-H, Gamse R, Pfannkuche H-J (1992) SDZ 205-557, a selective, surmountable antagonist for 5-HT_4 receptors in the isolated guinea-pig ileum. *Naunyn-Schmiedeberg's Arch Pharmacol* 345: 387-393.

Butler A, Elswood CJ, Burridge J, Ireland SJ, Kilpatrick GJ, Tyers MB (1990) The pharmacological characterization of 5-HT_3 receptors in three isolated preparations derived from guinea-pig tissues. *Br J Pharmacol* 101: 591-598.

Craig DA, Eglen RM, Walsh LKM, Perkins LA, Whiting RL, Clarke DE (1990) 5-Methoxytryptamine and 2-methyl-5-hydroxytryptamine-induced desensitization as a discriminative tool for the 5-HT_3 and putative 5-HT_4 receptors in the guinea pig ileum. *Naunyn-Schmiedeberg's Arch Pharmacol* 342: 9-16.

Dumuis A, Bouhelal R, Sebben M, Cory R, Bockaert J (1988) A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol Pharmacol* 34:880-887.

Dumuis A, Gozlan H, Sebben M, Ansanay H, Rizzi CA, Turconi M, Monferini E, Giraldo E, Schiantarelli P, Ladinsky H, Bockaert J (1992) Characterization of a novel 5-HT_4 receptor antagonist of the azabicycloalkyl benzimidazolone class: DAU 6285. *Naunyn-Schmiedeberg's Arch Pharmacol* 345: 264-269.

Elswood CJ, Bunce KT, Humphrey PPA (1991) Identification of putative 5-HT_4-receptors in guinea-pig ascending colon. *Eur J Pharmacol* 196: 149-155.

Elswood CJ, Bunce KT (1992) Investigation of the 5-HT receptor mediating relaxation in guinea-pig proximal colon. *J Pharm Pharmacol* 44: 264-266.

Fozard JR (1985) 5-Methoxytryptamine (5-MeOT) discriminates between excitatory neuronal 5-hydroxytryptamine (5-HT) receptors in the guinea-pig ileum. *J Pharmacol* 16: P 498.

Hasler WL, Heldsinger A, Owyang C (1991) Cisapride acts on muscarinic (glandular M_2) receptors to induce contractions of isolated gastric myocytes: mediation *via* a calcium-phosphoinositide pathway. *J Pharmacol Exp Ther* 259: 1294-1300.

Kilbinger H, Wolf D (1992) Effects of 5-HT_4-receptor stimulation on the basal and electrically evoked release of acetylcholine from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch Pharmacol* 345: 270-275.

Kilpatrick GJ, Bunce KT, Tyers MB (1990) 5-HT_3 Receptors. *Med Res Rev* 10: 441-475.

Kojima S (1991) Characterization of 5-hydroxytryptamine-induced relaxations of the guinea-pig proximal colon. *Arch Int Pharmacodyn* 313: 23-32.

Leysen JE (1990) Gaps and peculiarities in 5-HT_2 receptor studies. *Neuropsychopharmacology* 3: 361-369.

Meulemans AL, Schuurkes JAJ (1992) Is the action of cisapride on the guinea-pig ileum mediated *via* 5-HT_4 receptors? *Eur J Pharmacol* 212: 51-59.

Moriarty KJ, Higgs NB, Woodford M, Warhurst G, Turberg LA (1987) Inhibition of the effect of serotonin on rat ileal transport by cisapride: evidence in favour of the involvement of 5-HT_2-receptors. *Gut* 28: 844-848.

Müller-Lissner SA (1985) Cisapride in chronic constipation and laxative abuse. *Gastroenterology* 88: 1510-1516.

Müller-Lissner SA (1987) Treatment of chronic constipation with cisapride and placebo. *Gut* 28: 1033-1038.

Reyntjens A, Verlinden M, Aerts T (1986) Development and clinical use of the new gastrointestinal prokinetic drug cisapride (R 51619). *Drug Dev Res* 8: 251-265.

Sanger, GJ (1987) Activation of a myenteric 5-hydroxytryptamine-like receptor by metoclopramide. *J Pharm Pharmacol* 39: 449-453.

Schiavone A, Giraldo E, Giudici L, Turconi M, Sagrada A (1992) DAU 6285: a novel antagonist at the putative 5-HT_4 receptor. *Life Sci* 51: 583-592.

Scholtysik G, Imoto Y, Yatani A, Brown AM (1988) 5-Hydroxytryptamine antagonist ICS 205-930 blocks cardiac potassium, sodium and calcium currents. *J Pharmacol Exp Ther* 245: 773-778.

Schuurkes JAJ (1992) Cisapride and gastrointestinal motility in animal models. In: *Gastrointestinal dysmotility: focus on cisapride* (Heading RC, Wood JD Eds.), Raven Press Ltd., New York, pp 1-14.

Schuurkes JAJ, Van Nueten JM (1989) Stimulation of intrinsic cholinergic nerves and gastrointestinal motility. In: *Nerves and the gastrointestinal tract* (Singer MV, Goebell H Eds.), MTP Press Ltd., London, pp. 693-698.

Schuurkes JAJ, Van Nueten JM, Van Daele PGH, Reyntjens AJ, Janssen PAJ (1985) Motor-stimulating properties of cisapride on isolated gastrointestinal preparations of the guinea-pig. *J Pharmacol Exp Ther* 234: 775-783.

Taniyama K, Nakayama S, Takeda K, Matsuyama S, Shirakawa J, Sano I, Tanaka C (1991) Cisapride stimulates motility of the intestine *via* the 5-hydroxytryptamine receptors. *J Pharmacol Exp Ther* 258: 1098-1104.

Tonini M, Galligan JJ, North RA (1989) Effects of cisapride on cholinergic transmission and propulsive motility in the guinea-pig ileum. *Gastroenterology* 96: 1257-1264.

Tonini M, Rizzi CA, Manzo L, Onori L (1991) Novel enteric 5-HT_4 receptors and gastro-intestinal prokinetic action. *Pharmacol Res* 24: 5-14.

Tonini M, Candura SM, Onori L, Coccini T, Manzo L, Rizzi CA (1992) 5-hydroxytryptamine$_4$ receptor agonists facilitate cholinergic transmission in the circular muscle of guinea pig ileum: antagonism by tropisetron and DAU 6285. *Life Sci* 50: PL173-PL178.

Turconi M, Schiantarelli P, Borsini F, Rizzi CA, Ladinsky H, Donetti A (1991) Azabicycloalkyl benzimidazolones: Interaction with serotonergic 5-HT_3 and 5-HT_4 receptors and potential therapeutic implications. *Drugs Fut* 16: 1011-1026.

Van Nueten JM, Schuurkes JAJ (1989) 5-HT_3 serotonergic mechanisms and stimulation of gastrointestinal (GI) motility by cisapride. *Br J Pharmacol* 96 (suppl): 331P.

Wade PR, Mawe GM, Branchek, TA, Gershon MD (1991) Use of stereoisomers of zacopride to analyze actions of 5-hydroxytryptamine on enteric neurons. *Am J Physiol* 260: G80-G90.

Chapter 5

Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*

M.R. Briejer, L.M.A. Akkermans, A.L. Meulemans,
R.A. Lefebvre and J.A.J. Schuurkes

Slightly modified: *Br J Pharmacol* (1992) 107: 756-761

Abstract. In the guinea-pig colon ascendens, 5-hydroxytryptamine (5-HT) induces contractions, mediated by 5-HT_2, 5-HT_3 and 5-HT_4 receptors, and relaxations, through a 5-HT_1 receptor subtype, that triggers the release of an inhibitory neurotransmitter. Nitric oxide (NO) is one of the main candidates of NANC inhibitory neurotransmission in the gut. The aim of this study was to establish whether NO is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens.

Antagonists to block the contractile responses to 5-HT *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors were present throughout the experiments and methacholine was administered to elevate tone. Under these conditions, 5-HT concentration-dependently induced relaxations from 10 nM onwards (EC_{50} = 258 (172-387) nM). The relaxations were inhibited by metergoline (10 nM) and methiothepine (0.1 μM) and abolished by TTX (0.3 μM). Guanethidine (3 μM) did not affect them. N^G-nitro-L-arginine (L-NNA) inhibited the responses to 5-HT (IC_{50} = 18.7 (13.3-26.3) μM); at the highest 5-HT concentration a maximum inhibition of about 75 % was observed with 0.3 mM L-NNA. This inhibition was reversed with L-arginine. Relaxations to glyceryl trinitrate (GTN) were not inhibited by L-NNA. Haemoglobin (30 μM) inhibited the relaxations to 5-HT and GTN, but not those to isoproterenol (ISO). Methylene blue (10 μM) inhibited the relaxations to 5-HT but did not affect those caused by GTN or ISO.

It is concluded that 5-HT induces relaxations that involve NO. We also confirmed that 5-HT induces these relaxations *via* (a) 5-HT_1 receptor subtype(s), located on neurons.

Introduction

Inhibitory enteric neurons play an important role in gut motility. They are involved in reflex relaxation of the lower esophageal and the internal anal sphincter, the receptive and adaptive relaxation of the stomach and the descending inhibition during intestinal peristalsis. The exact mechanisms underlying these phenomena remain to be elucidated.

In isolated colon ascendens preparations of the guinea-pig, 5-hydroxytryptamine (5-HT), a neurotransmitter of the enteric nerves, induces both contractions and relaxations (Costa & Furness, 1979). It has previously been shown, that the contractions are mediated *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors (Briejer *et al.*, 1991; Sato *et al.*, 1991, Elswood *et al.*, 1991), and the relaxations through a 5-HT-receptor different from 5-HT_2, 5-HT_3 or 5-HT_4 receptors (Briejer *et al.*, 1991; Sato *et al.*, 1991) but sensitive to 5-HT_1 receptor antagonists (Kojima, 1991; Elswood & Bunce, 1992). Since TTX abolished these relaxations, they were presumed to be mediated by nerves (Gershon, 1967). In the guinea-pig proximal (Costa & Furness, 1979) and distal colon (Onori *et al.*, 1984), 5-HT-evoked relaxations were not affected by incubation with guanethidine, which suggests that the relaxations are mediated by the intrinsic NANC (non-adrenergic, non-cholinergic) inhibitory neurons. Several compounds have been proposed as a neurotransmitter of these NANC inhibitory neurons: ATP (Burnstock, 1972), vasoactive

intestinal peptide (VIP) (Makhlouf, 1985) and nitric oxide (NO) (Moncada *et al.*, 1991).

Evidence for NO as a transmitter of inhibitory motor neurons in the gastrointestinal tract have been obtained by several authors in various tissues: canine ileocolonic junction (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990), canine terminal ileum (Bogers *et al.*, 1991), guinea-pig ileum (Osthaus & Galligan, 1992), guinea-pig stomach (Desai *et al.*, 1991; Meulemans *et al.*, 1993), rat gastric fundus (Li & Rand, 1990), rat stomach (Lefebvre *et al.*, 1992), opossum internal anal sphincter (Rattan & Chadker, 1992; Tøttrup *et al.*, 1992), human ileum (Maggi *et al.*, 1991), human sigmoid colon and internal anal sphincter (Burleigh, 1992).

Much of this evidence is based on the use of compounds that selectively modify the "nitrergic" neurotransmission. NO is formed from L-arginine by NO synthase (Palmer *et al.*, 1988), which has been immunohistochemically demonstrated in the myenteric plexus of the rat (Bredt *et al.*, 1990). NO readily diffuses through the presynaptic membrane into the target cell, where it is thought to activate the soluble guanylate cyclase, thus giving rise to increased cyclic GMP-levels (Arnold *et al.*, 1977; Feelish & Noack, 1987a; Moncada *et al.*, 1991; McCall & Vallance, 1992). This L-arginine-NO-pathway can be inhibited at several levels. Analogues of L-arginine inhibit the synthesis of NO (Moncada *et al.*, 1991; McCall & Vallance, 1992). N^G-nitro-L-arginine has been shown to be a potent inhibitor of the NO-synthesis (Moore *et al.*, 1990; Mülsch & Busse, 1990). Haemoglobin captates NO after it has diffused out of the neuron, which will prevent it from diffusing into the smooth muscle cells (Martin *et al.*, 1985; Kelm & Schrader, 1990). The dye and oxidant methylene blue selectively inhibits the target of NO, the soluble guanylate cyclase (Gruetter *et al.*, 1981; Martin *et al.*, 1985) which is present in the smooth muscle cells.

The aim of this study was to establish whether NO is involved in relaxations upon 5-HT administration of the guinea-pig colon ascendens.

Materials and methods

Tissue preparation

Dunkin-Hartley guinea-pigs of both sexes, weighing 400-600 g, were killed by cervical dislocation. The ascending colon was removed, and the luminal contents were washed out with De Jalon solution (mM: KCl 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, NaCl 155, glucose 2.78). The mesentery was carefully removed. Starting at the proximal end, about 1 cm distal from the caecum, the colon was

divided into four segments of circa 2.5 cm. These intact segments were individually mounted vertically for isotonic measurement into an tissue bath containing 20 ml De Jalon's solution. This solution was kept at 37 °C and gassed with 95% O_2, 5 % CO_2. The strips were subjected to a preload of 2 g and allowed to stabilize for half an hour. Ketanserin (0.3 μM) and tropisetron (ICS 205-930, 3 μM) were continuously present in the De Jalon's solution during all experiments to block contractile responses mediated by 5-HT_2, 5-HT_3 and 5-HT_4 receptors. After stabilization, the response of the longitudinal muscle to 0.3 μM methacholine was measured. After washing and 10 min stabilization, the procedure was repeated in order to stabilize the response of the tissue.

Protocols

For the construction of concentration-response curves, agonists and antagonists were applied directly to the tissue bath (added volume ≤ 3 % of tissue bath volume). Methacholine (0.3 μM) was added to the bath in order to precontract the muscle. The mean decrease in length was 5.78 ± 0.24 mm.

5-Hydroxytryptamine. Since pilot experiments had revealed that relaxations to 5-HT are subjected to tachyphylaxis, non-cumulative concentration-response curves were established, beginning 10 min after the administration of methacholine. The size of the 5-HT-induced relaxation was measured as the maximum relaxation induced during an incubation period of approximately 2 min. Then, the agonists were washed out by replacing the bathing fluid twice. Stabilization of 10 min was allowed. This 20 min dosing cycle was repeated applying ascending concentrations of 5-HT. The response to repetitive administration of methacholine was perfectly stable throughout the experiment. On each segment, first a concentration-response curve to 5-HT was constructed in the absence of an antagonist (ketanserin and tropisetron present) (= first series). Consecutively, the measurements were repeated in the presence of an antagonist (= second series). Antagonists were added 10 min prior to the methacholine-induced precontraction, and were re-added directly after each washout. The antagonists had no effect on the methacholine-induced contractions, except for methiothepin. When L-arginine was tested against N^G-nitro-L-arginine, they were added together. Four segments were taken per animal; each fourth strip was utilized as a control, i.e. vehicle control was added during the second series. In these control strips, the concentration-response curve to 5-HT was perfectly reproducible as compared to the curves of the first series. All responses were expressed as a percentage of the

relaxation induced by 5-HT 10 μM of the first series for each individual segment. 5-HT (10 μM) induced relaxations of 3.8 ± 0.2 mm (n = 32; mean ± s.e.m.).

Glyceryl trinitrate and isoproterenol. For the construction of concentration-response curves to glyceryl trinitrate and isoproterenol, the compounds were added in a cumulative fashion, 10 min after precontraction to methacholine-addition (0.3 μM). After washing and stabilization for 30 min (washing every 10 min), a second concentration-response curve was established in the presence of an antagonist. The responses induced by glyceryl trinitrate or isoproterenol were expressed relative to the response to either glyceryl trinitrate 10 μM or isoproterenol 3 μM of the first series for each segment. The relaxations induced by these concentrations were respectively 7.6 ± 0.5 mm (n = 18) and 10.5 ± 1.0 mm (n = 12).

Chemicals

Methiothepin maleate (Hoffman-La Roche, Switzerland), methacholine HCl (E.Merck, Germany), 5-hydroxytryptamine HCl, tetrodotoxin (Serva, Germany), bovine haemoglobin (Sigma, Belgium), glyceryl trinitrate (Federa, Belgium), metergoline (Gruppo Montedison Farmitalia, Italy), isoproterenol tartrate, methylene blue, N^G-nitro-L-arginine and L-arginine (Janssen Chimica, Belgium) were added to the tissue bath solution in volumes less than 3% of the bath volumes. Ketanserin tartrate (Janssen Pharmaceutica, Belgium) and tropisetron HCl (ICS 205-930; Sandoz, Switzerland) were continuously present in the De Jalon's solution. All compounds were dissolved in distilled water, except for 5-hydroxytryptamine (stock containing ascorbic acid 0.25 μM). This vehicle had no effect on the tone of the strips. Glyceryl trinitrate was further diluted with distilled water from a stock solution of 1 % in ethanol. The solution of haemoglobin was always freshly prepared, kept on ice during the experiment and protected from light. The stock solution of TTX was prepared in advance and kept frozen (-25 °C) in aliquots of 100 μM. It was kept on ice during the experiment.

Statistical analysis

For graphical representation means ± standard error of the means were calculated. EC_{50} and IC_{50} values were determined by linear regression analysis. Differences between mean values were tested using the two-tailed Student's *t*-test for unpaired observations. Values of $P<0.05$ were considered to be statistically different.

Results

5-HT induced concentration-dependent relaxations from 10 nM onwards (Figure 5.1). The relaxations were fast in onset. In about 1 out of 3 preparations, the relaxations induced by 5-HT in the concentration range of 0.3 μM to 3 μM were multiphasic, i.e. an initial relaxation followed by a quick small contraction and consecutively a larger, more sustained relaxation.

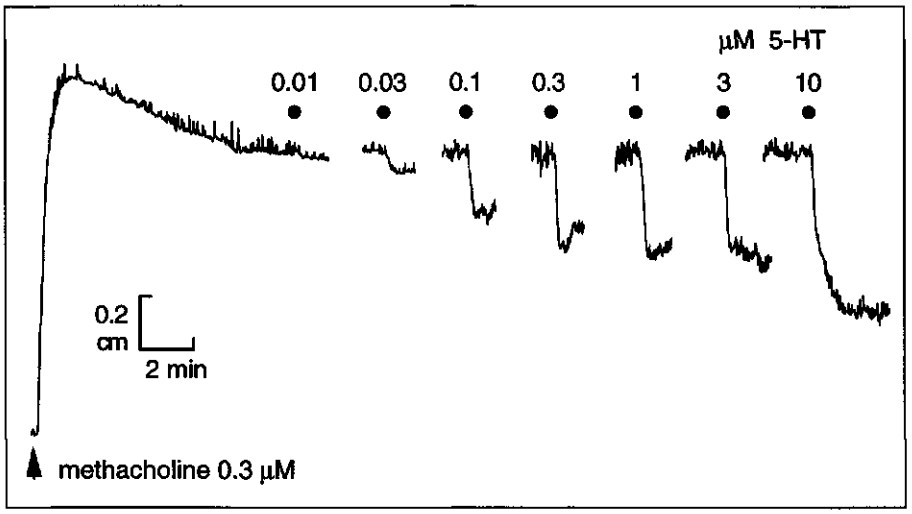


Figure 5.1 Tracing of relaxations of the guinea-pig colon ascendens evoked by non-cumulative concentrations of 5-HT. The tone of the preparation was elevated by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT_2, 5-HT_3 and 5-HT_4 receptors.

The concentration-response curve to control 5-HT appeared non-monophasic, starting a second phase at about 1.0 μM (Figure 5.2). The EC_{50} value calculated over the entire concentration-response curve to 5-HT was 258 (172-387) nM. TTX (0.3 μM) contracted the strips (18.4 ± 3.8 % (n = 7) of the methacholine-induced contractions), and it abolished the relaxations to 5-HT 10 μM (n = 6, not shown). The 5-HT_1 receptor antagonist metergoline (10 nM) inhibited the relaxations to 5-HT (Figure 5.2). Methiothepin (0.1 μM) also inhibited the relaxations to 5-HT (by about 45-70 % depending on the applied 5-HT concentration) but it inhibited the methacholine-induced contractions as well (by about 25 %).

Guanethidine (3 μM) did not affect the concentration-response curve to 5-HT (n = 4, results not shown).

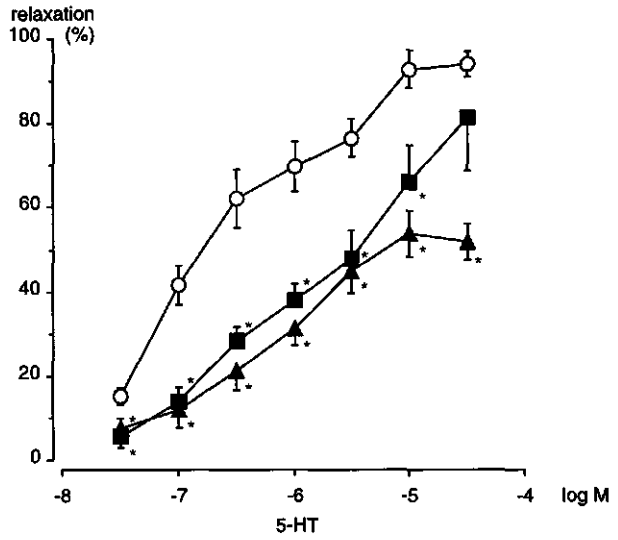


Figure 5.2 Concentration-response curves to 5-HT: control (O), or in the presence of metergoline 10 nM (■) or methiothepin 0.1 μM (▲). The tone of the preparation was elevated by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT_2, 5-HT_3 and 5-HT_4 receptors. Values (n = 6) that are significantly different from control values ($P<0.05$) are marked with an asterisk. The relaxations were expressed as a percentage of the relaxations to 5-HT 10 μM.

NO-antagonists

N^G-nitro-L-arginine concentration-dependently depressed the concentration-response curve to 5-HT, the inhibitory effect being statistically significant from 10 μM N^G-nitro-L-arginine onwards (Figure 5.3). The IC_{50} of the inhibitory effects of N^G-nitro-L-arginine against 5-HT-induced relaxations was 18.7 (13.3-26.3) μM (calculated at 5-HT 0.3 μM). N^G-nitro-L-arginine 0.3 mM abolished relaxations to 5-HT up to 0.3 μM. However, the relaxations caused by 5-HT > 0.3 μM were not completely blocked: about 25 % of the control response remained (Figure 5.3). The high concentrations of N^G-nitro-L-arginine elevated the tone of the preparation slightly (27.2 ± 4.5 % (n = 6) of the methacholine-induced contractions). L-Arginine (0.1 mM) did not affect the concentration-response curve to 5-HT (n = 3, results not shown). L-Arginine (0.3 mM) did completely reverse the

inhibition by N^G-nitro-L-arginine (10 μM) against 5-HT (0.1 μM)-induced relaxations (n = 6, results not shown).

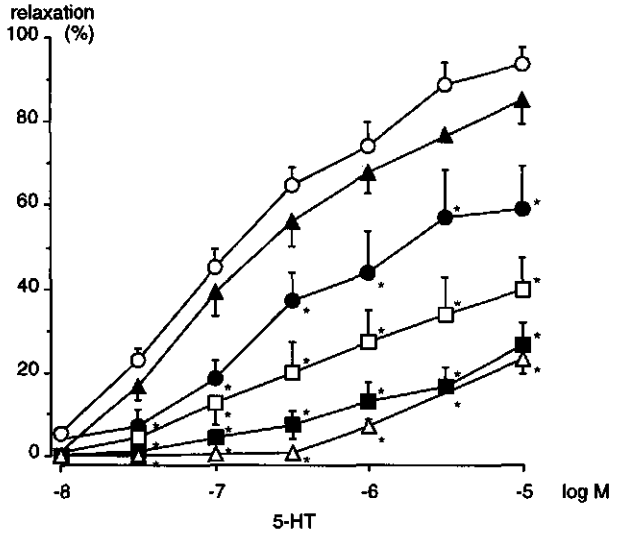


Figure 5.3 Concentration-response curves to 5-HT: control (O), or in the presence of N^G-nitro-L-arginine 3 μM (▲), 10 μM (●), 30 μM (□), 0.1 mM (■) and 0.3 mM (Δ). The tone of the preparation was elevated by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT_2, 5-HT_3 and 5-HT_4 receptors. Values (all n = 6; control n = 12) that are significantly different from control values ($P<0.05$) are marked with an asterisk.The relaxations were expressed as a percentage of the relaxations to 5-HT 10 μM.

Glyceryl trinitrate concentration-dependently induced relaxations from 3 nM onwards (Figure 5.4) which were fast in onset. TTX (0.3 μM), N^G-nitro-L-arginine (0.1 mM) and L-arginine (0.1 mM) did not inhibit the responses to glyceryl trinitrate (Figure 5.4).

Haemoglobin 10 μM tended to inhibit the 5-HT-induced relaxations, whereas haemoglobin 30 μM significantly inhibited them (Figure 5.5, *left panel*). Isoproterenol relaxed the preparations from 1 nM onwards. The relaxations were not fast in onset. Haemoglobin 30 μM did not affect the concentration-response curves to isoproterenol (Figure 5.5, *middle panel*). In contrast, it depressed the concentration-response curve to glyceryl trinitrate (Figure 5.5, *right panel*). Haemoglobin slightly contracted the muscle (4.6 ± 1.6 % of methacholine-induced contractions (n = 10)).

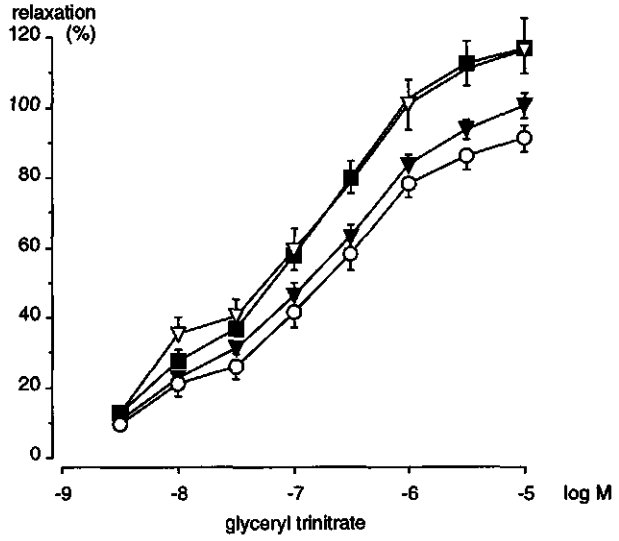


Figure 5.4 Concentration-response curves to glyceryl trinitrate in the absence of an antagonist (O) or in the presence of L-arginine 0.1 mM (▼), N^G-nitro-L-arginine 0.1 mM (■) or TTX 0.3 μM (●). The tone of the preparation was elevated by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT_2, 5-HT_3 and 5-HT_4 receptors. All values n = 6. The relaxations were expressed as a percentage of the relaxations to glyceryl trinitrate 10 μM.

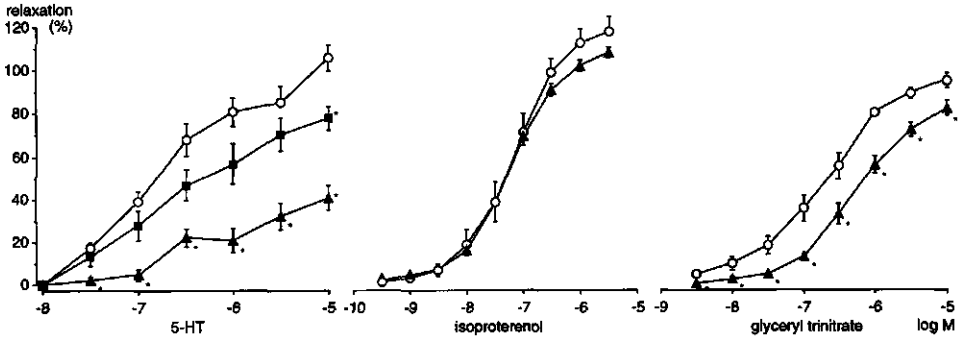


Figure 5.5 Concentration-response curves to 5-HT (*left panel*), isoproterenol (*middle panel*) and glyceryl trinitrate (*right panel*): controls (O), or in the presence of haemoglobin 10 μM (■) and 30 μM (▲). The tone of the preparation was elevated by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT_2, 5-HT_3 and 5-HT_4 receptors. Values (n = 6) that are significantly different from control values ($P<0.05$) are marked with an asterisk. The relaxations to 5-HT, isoproterenol or glyceryl trinitrate were expressed as a percentage of the relaxations to 5-HT 10 μM, isoproterenol 3 μM or glyceryl trinitrate 10 μM respectively.

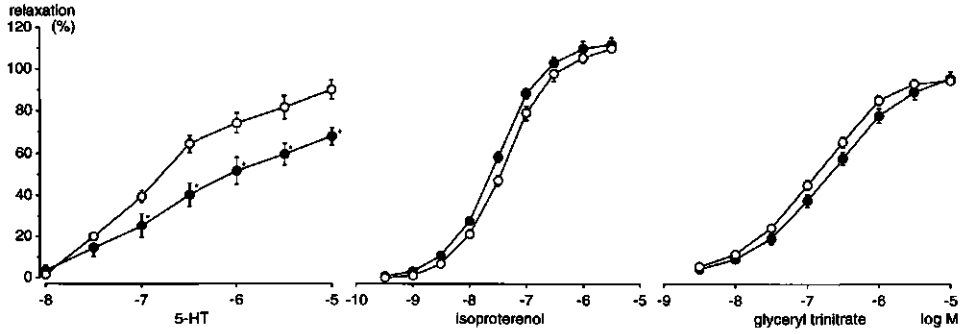


Figure 5.6 Concentration-response curves to 5-HT (*left panel*), isoproterenol (*middle panel*) and glyceryl trinitrate (*right panel*): controls (O), or in the presence of methylene blue 10 μM (●). The tone of the preparation was elevated by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT_2, 5-HT_3 and 5-HT_4 receptors. Values (n = 6) that are significantly different from control values ($P<0.05$) are marked with an asterisk. The relaxations to 5-HT, isoproterenol or glyceryl trinitrate were expressed as a percentage of the relaxations to 5-HT 10 μM, isoproterenol 3 μM or glyceryl trinitrate 10 μM respectively.

Methylene blue (10 μM) inhibited the responses to 5-HT from 0.1 μM onwards (Figure 5.6, *left panel*). The concentration-response curves to isoproterenol or glyceryl trinitrate however were not affected (Figure 5.6, *middle* and *right panels*). Methylene blue (10 μM) contracted the muscle slightly (6.2 ± 2.1 % of the methacholine-induced contractions (n = 6)). Higher concentrations of methylene blue could not be tested because they inhibited the methacholine-induced contractions seriously.

Discussion

The selective 5-HT_1 receptor antagonists methiothepin and metergoline both inhibited the relaxations caused by 5-HT. The selective 5 HT_2 receptor antagonist ketanserin and the 5-HT_3 and 5-HT_4 receptor antagonist tropisetron were present throughout our experiments. It therefore seems likely that, according to the criteria for 5-HT receptor classification as proposed by Bradley *et al* . (1986), the relaxations to 5-HT are mediated via 5-HT_1 receptors. Kojima (1991) and Elswood & Bunce (1992) also found a 5-HT_1 receptor to be involved in 5-HT-induced relaxations in the guinea-pig colon. It was however not established which 5-HT_1 receptor subtype is concerned. In intestinal tissues of the guinea-pig, rat and rabbit, receptors with 5-HT_{1A} (Fozard & Kilbinger, 1985; Matsuyama *et al.*, 1990; Wade

et al., 1991; Galligan, 1992), putative 5-HT_{1P} (Mawe *et al.*, 1986; Branchek *et al.*, 1988; Wade *et al.*, 1991), 5-HT_{1C} and 5-HT_{1D} (Baez *et al.*, 1991; Kalkman & Fozard, 1991) characteristics have been demonstrated. Further studies are required to elucidate which 5-HT_1 receptor subtype is involved in the 5-HT-induced relaxations of the guinea-pig colon.

TTX abolished relaxant responses to 5-HT, which confirms that nerves are involved. Since guanethidine did not affect the 5-HT-induced relaxations (Costa & Furness, 1979; present results), it is not likely that noradrenaline is the inhibitory neurotransmitter involved. In the canine terminal ileum and ileocolonic junction, it has been demonstrated that 5-HT-induced relaxations are mediated by NO (Bogers *et al.*, 1991). In our study, evidence is obtained which strongly suggests that NO is involved in 5-HT-induced relaxations in the guinea-pig colon ascendens as well. This evidence consists of three sets of observations.

Firstly, the L-arginine analogue N^G-nitro-L-arginine, which is known to be a potent inhibitor of NO-synthase (Moore *et al.*, 1990; Mülsch & Busse, 1990), inhibited the relaxations to 5-HT but not those to glyceryl trinitrate. Furthermore, the inhibition could be reversed by L-arginine. These data suggest that NO is involved in the 5-HT-induced relaxations.

Secondly, both the relaxations to glyceryl trinitrate and 5-HT but not those to isoproterenol were inhibited by haemoglobin. Although the exact mechanism of action is still under debate, glyceryl trinitrate is thought to react with thiol groups of co-factors (cysteine) of the soluble guanylate cyclase, which yields the generation of NO (Ignarro *et al.*, 1981; Feelish *et al.*, 1988). NO in turn activates the soluble guanylate cyclase to increase the intracellular cyclic GMP content (Feelish & Noack, 1987a; b). Haemoglobin is a scavenger of NO (Martin *et al.*, 1985; Kelm & Schrader, 1990). It has been shown that haemoglobin does not bind or inactivate glyceryl trinitrate and that it does not affect the reaction between glyceryl trinitrate and the thiols (Martin *et al.*, 1986; Feelish & Noack, 1987b). In our experiments, neither TTX nor N^G-nitro-L-arginine or L-arginine inhibited the relaxations to glyceryl trinitrate, which is in good agreement with the above mechanism of action. Due to its large size, haemoglobin is likely to be confined to the extracellular space. Since NO readily crosses cell membranes, haemoglobin might avidly bind the extracellular NO which will lower its extracellular concentration, thus increasing the net rate of diffusion of NO out of the cell (Martin *et al.*, 1985; 1986). This might explain the observed inhibition by haemoglobin against the relaxations to glyceryl trinitrate. Other authors have also found haemoglobin to inhibit relaxations to glyceryl trinitrate: Martin *et al.* (1985, 1986; rabbit aorta), Boeckxstaens *et al.* (1990; canine ileocolonic junction), Bogers *et al.*

(1991; canine terminal ileum and ileocolonic junction). Isoproterenol, on the other hand, induces relaxations through an increase of intracellular cyclic AMP-content (Martin *et al.*, 1985). Hence, the inhibition by haemoglobin of the relaxations to 5-HT and glyceryl trinitrate, but not isoproterenol, strongly suggests that NO is involved in the action of 5-HT.

Thirdly, the relaxations to 5-HT were inhibited by the oxidant methylene blue, whereas the responses to glyceryl trinitrate and isoproterenol were not. Methylene blue is a selective inhibitor of the target of NO, the soluble guanylate cyclase. (Gruetter *et al.*, 1981; Martin *et al.*, 1985). Hence, it would be expected that the responses to glyceryl trinitrate would also be inhibited, as it does so in other tissues (Gruetter *et al.*, 1981; Martin *et al.*, 1985; Desai *et al.*, 1991). We do not have an explanation for this observation. The inhibition of the responses to 5-HT but not to isoproterenol however provides additional proof for the involvement of NO.

Methylene blue, N^G-nitro-L-arginine, hemoglobine and also the neurotoxin TTX all contracted the strips. This might indicate that NO constantly leaks from enteric inhibitory nerves in small quantities.

The concentration-response curve to 5-HT does not seem to be monophasic. Furthermore, N^G-nitro-L-arginine was not able to abolish the responses to 5-HT. Both these findings suggest that, at higher concentrations of 5-HT, inhibitory endogenous substances might be co-released with NO. Whether other candidates for inhibitory NANC-neurotransmission, which implicates VIP and ATP, are involved, needs further investigation.

It is concluded that in the guinea-pig colon ascendens NO is involved in the relaxation that is induced by 5-HT. Our studies confirm that the relaxations are mediated by 5-HT_1 receptor subtype. The involvement of VIP and/or ATP can not be excluded.

References

Arnold WP, Mittal CK, Katsuki S, Murad F (1977) Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci USA* 74: 3203-3207.

Baez M, Yu L, Cohen L (1991) Is contraction to serotonin mediated via 5-HT_{1C}-receptor activation in rat stomach fundus? In: *Serotonin: molecular biology, receptors and functional effects.* (Fozard JR, Saxena PR Eds.) Birkhäuser Verlag, Basle Switzerland: pp. 144-152.

Boeckxstaens GE, Pelckmans PA, Bult H, De Man JG, Herman AG, Van Maercke YM (1990) Non-adrenergic non-cholinergic relaxation mediated by nitric oxide in the canine ileocolonic junction. *Eur J Pharmacol* 190: 239-246.

Bogers JJ, Pelckmans PA, Boeckxstaens GE, De Man JG, Herman AG, Van Maerke YM (1991) The role of nitric oxide in serotonin-induced relaxations in the canine terminal ileum and ileocolonic junction. *Naunyn-Schmiedeberg's Arch Pharmacol* 344: 716-719.

Bradley PB, Engel G, Feniuk W, Fozard JR, Humphrey PPA, Middlemiss DN, Mylecharane EJ, Richardson BP, Saxena PR (1986) Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology* 6: 563-576.

Branchek TA, Mawe GM, Gershon MD (1988) Characterization and localization of a peripheral neural 5-hydroxytryptamine receptor subtype (5-HT_{1P}) with a selective agonist, ^{3}H-5-hydroxyindalpine. *J Neurosci 8*: 2582-2595.

Bredt DS, Hwang PM, Snyder SH (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 347: 768-770.

Briejer MR, Meulemans AL, Schuurkes JAJ (1991) Site related distribution of 5-HT-receptors in guinea-pig colon ascendens. *Pflügers Arch (Eur J Physiol)* 419: R 5.

Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM, Herman AG (1990) Nitric oxide as an inhibitory non-adrenergic non cholinergic neurotransmitter *Nature* 345: 346-347.

Burleigh DE (1992). N^G-nitro-L-arginine reduces nonadrenergic, noncholinergic relaxations of human gut. *Gastroenterology* 102: 679-683.

Burnstock G (1972) Purinergic nerves. *Pharmacol Rev* 24: 509-581.

Costa M, Furness JB (1979) The sites of action of 5-hydroxytryptamine in nerve-muscle preparations from the guinea-pig small intestine and colon. *Br J Pharmacol* 65: 237-248.

Desai KM, Sessa WC, Vane JR (1991) Involvement of nitric oxide in the reflex relaxation of the stomach to accommodate food or fluid. *Nature* 351: 477-479.

Elswood CJ, Bunce KT, Humphrey PPA (1991) Identification of putative 5-HT_4-receptors in guinea-pig ascending colon. *Eur J Pharmacol* 196: 149-155.

Elswood CJ & Bunce KT (1992) Investigation of the 5-HT receptor mediating relaxation in guinea-pig proximal colon. *J Pharm Pharmacol* 44: 264-266.

Feelisch M, Noack E (1987a). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur J Pharmacol* 139: 19-30.

Feelisch M, Noack E (1987b) Nitric oxide (NO) formation from nitrovasodilators occurs independently of haemoglobin or non-heme iron. *Eur J Pharmacol* 142: 465-469.

Feelisch M, Noack E, Schröder H (1988) Explanation of the discrepancy between the degree of organic nitrate decomposition, nitrite formation and guanylate cyclase stimulation. *Eur Heart J* 9 (suppl. A): 57-62.

Fozard JR, Kilbinger H (1985) 8-OH-DPAT inhibits transmitter release from guinea-pig enteric cholinergic neurones by activating 5-HT_{1A} receptors. *Br J Pharmacol* 86: 601 P.

Galligan JJ (1992) Differential inhibition of cholinergic and noncholinergic neurogenic contractions by 5-hydroxytryptamine$_{1A}$ receptor agonists in guinea pig ileum. *J Pharmacol Exp Ther* 260: 306-312.

Gershon MD (1967) Effects of tetrodotoxin in innervated smooth muscle preparations. *Br J Pharmacol Chemother* 29: 259-279.

Gruetter CA, Kadowitz PJ, Ignarro LJ (1981) Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerin, sodium nitrite and amyl nitrite. *Can J Physiol Pharmacol* 59: 150-156.

Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PD, Gruetter CA (1981) Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* 218: 739-749.

Kalkman HO, Fozard JR (1991) Further definition of the 5-HT-receptor mediating contraction of rat stomach fundus: relation to 5-HT_{1D} recognition sites. In: *Serotonin: molecular biology, receptors and functional effects* (Fozard JR, Saxena PR) Birkhäuser Verlag, Basle, Switzerland: pp. 144-152.

Kelm M, Schrader J (1990) Control of coronary vascular tone by nitric oxide. *Circ Res* 66: 1561-1575.

Kojima S (1991) Characterization of 5-hydroxytryptamine-induced relaxations of guinea-pig proximal colon. *Arch Int Pharmacodyn* 313: 23-32.

Lefebvre RA, Hasrat J, Gobert A (1992) Influence of N^G-nitro-L-arginine methyl ester on vagally induced gastric relaxation in the anaestetized rat. *Br J Pharmacol* 105: 315-320.

Li CG, MJ Rand (1990) Nitric oxide and vasoactive intestinal polypeptide mediated non-adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur J Pharmacol* 191: 303-309.

Maggi CA, Barbanti G, Turini D,Giuliani S (1991) Effect of N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NOARG) on non-adrenergic non-cholinergic relaxation in the circular muscle of the human ileum. *Br J Pharmacol* 103: 1970-1972.

Makhlouf GM Enteric neuropeptides: role in neuromusculair activity of the gut. *Trends Pharmacol Sci* (1985) 6: 214-218.

Martin W, Villani GM, Jothianandan D, Furchgott RF (1985) Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by haemoglobin and by methylene blue in the rabbit aorta. *J Pharmacol Exp Ther* 232: 708-716.

Martin W, Smith JA, White DG (1986) The mechanisms by which haemoglobin inhibits the relaxation of rabbit aorta induced by nitrovasodilators, nitric oxide, or bovine retractor penis inhibitory factor. *Br J Pharmacol* 89: 563-571.

Matsuyama S, Taniyama K, Takeda K, Tanaka C (1990) 5-Hydroxytryptamine receptor subtypes involved in the intestinal motility. *Eur J Pharmacol* 183: 2201-2202.

Mawe GM, Branchek TA, Gershon MD (1986) Peripheral neural serotonin receptors: identification and characterization with specific antagonists and agonists. *Proc Natl Acad Sci USA* 83, 9799-9803.

McCall T, Vallance P (1992). Nitric oxide takes centre-stage with newly defined roles. *Trends Pharmacol Sci* 13: 1-6.

Meulemans AL, Helsen LF, Schuurkes JAJ (1993) Role of NO in vagally-mediated relaxations of guinea-pig stomach. *Naunyn-Schmiedeberg's Arch Pharmacol* 347: 225-230..

Moncada S, Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43: 109-142.

Moore PK, al-Swayeh OA, Chong NWS, Evans RA, Gibson A (1990) L-N^G-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br J Pharmacol* 99: 408-412.

Mülsch A, Busse R (1990) N^G-nitro-L-arginine (N^5-[imino-(nitroamino)-methyl]-L-ornithine) impairs endothelium-dependent dilatations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmiedeberg's Arch Pharmacol* 341: 143-147.

Onori L, Saltarelli P, Marcoli M, Lecchini S, Frigo GM, Tonini M (1984) 5-Hydroxytryptamine desensitization fails to modify GABA-induced inhibitory responses in the guinea-pig colon. *J Pharm Pharmacol* 36: 406-408.

Osthaus LE, Galligan JJ (1992) Antagonists of nitric oxide synthesis inhibit nerve-mediated relaxations of longitudinal muscle in guinea pig ileum. *J Pharmacol Exp Ther* 260: 140-145.

Palmer RMJ, Ashton DS, Moncada S (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333: 664-666.

Rattan S, Chakder S (1992) Role of nitric oxide as a mediator of internal anal sphincter relaxation. *Am J Physiol* 262: G107-G112.

Sato N, Keiichiro H, Setoguchi M (1991) The pharmacological characteristics of 5-HT_4 receptors in the isolated guinea-pig ascending colon. Proceedings of the 6th annual meeting of the Japanese Pharmacological Society, March 24-27, 1991, Kobe, Japan, *Jap J Pharmacol*: 393P.

Tøttrup A, Glavind EB, Svane D (1992) Involvement of the L-arginine-nitric oxide pathway in internal anal sphincter relaxation. *Gastroenterology* 102: 409-415.

Wade PR, Mawe GM, Branchek TA, Gershon MD (1991) Use of stereoisomers of zacopride to analyze actions of 5-hydroxytryptamine on enteric neurons. *Am J Physiol* 260: G80-G90.

Chapter 6

5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide

M.R. Briejer, L.M.A. Akkermans, A.L. Meulemans, R.A. Lefebvre and J.A.J. Schuurkes

slightly modified: *Naunyn-Schmiedeberg's Arch Pharmacol* (1995) 351: 126-135

Abstract. In the guinea-pig proximal colon, 5-hydroxytryptamine (5-HT) relaxes the longitudinal muscle by stimulating neuronal 5-HT receptors, which induces the release of nitric oxide (NO). It was investigated whether the inhibitory neurotransmitters adenosine 5'-triphosphate (ATP) and/or vasoactive intestinal polypeptide (VIP) could be involved as well.

Antagonists to block the contractile responses to 5-HT *via* 5-HT_2, 5-HT_3 or 5-HT_4 receptors were present throughout the experiments and methacholine was administered to precontract the strips. ATP, VIP and 5-HT induced concentration-dependent relaxations, in case of 5-HT yielding a non-monophasic concentration-response curve. Tetrodotoxin (TTX; 300 nM), N^G-nitro-L-arginine (L-NNA; 100 μM) and the combination treatment did not inhibit the relaxations induced by VIP (up to 0.3 μM) or 0.3-3 μM ATP but reduced those by 10 μM ATP. Suramin (300 μM) strongly inhibited the relaxations to ATP and VIP. L-NNA and suramin also inhibited the relaxations to 5-HT. In the presence of L-NNA (100 μM), suramin did not significantly inhibit the relaxations to 5-HT. Suramin did not affect the relaxations to isoprenaline, nitroglycerin or exogenous NO (1 μM), demonstrating its specificity. Apamin (30 nM) inhibited both the relaxations to ATP (by 70-100 %) and to 5-HT; relaxations to isoprenaline were partially inhibited, indicating a non-specific component in the inhibitory action of apamin. However, relaxations to exogenous VIP (up to 0.3 μM), NO (1 μM) and to nitroglycerin were not inhibited. In the presence of L-NNA (100 μM), apamin inhibited the relaxations to 5-HT only at 30 μM. α,β-methylene-ATP (α,β-Me-ATP; 100 μM) did not desensitize the responses to ATP. Reactive blue 2 affected the relaxations to isoprenaline at concentrations necessary to significantly inhibit the relaxations to ATP (i.e. from 10 μM onwards). Thus, it was not possible to test either α,β-Me-ATP or reactive blue 2 against the relaxations to 5-HT. α-Chymotrypsin (0.015 $mg.ml^{-1}$) and trypsin (0.005 $mg.ml^{-1}$) almost abolished the relaxations to VIP, but did not affect those to isoprenaline and 5-HT. The VIP receptor antagonists [p-Cl-D-Phe^6,Leu^{17}]VIP (1 μM) and $VIP_{10\text{-}28}$ (1 and 3 μM) did not affect the concentration-response curve to VIP and were hence not tested against 5-HT. Phosphoramidon (1 μM) had no effect on the relaxations to VIP or 5-HT.

It can be concluded that on the guinea-pig colon longitudinal muscle, VIP and ATP induce relaxation *via* a direct effect of the smooth muscle, not involving NO. 5-HT-induced relaxations are mediated by NO as well as by a substance which is sensitive to inhibition by suramin and apamin. It is suggested that this substance is ATP and not a peptide.

Introduction

5-Hydroxytryptamine (5-HT; serotonin), a neurotransmitter in the enteric nervous system, causes a complex response of the longitudinal muscle of the proximal guinea-pig colon. 5-HT induces contractions through the stimulation of 5-HT_2, 5-HT_3 and 5-HT_4 receptors (Briejer *et al.*, 1993), as well as relaxations. These relaxations are abolished by tetrodotoxin (TTX), a blocker of neuronal conductance, and are inhibited by 5-HT_1 receptor antagonists (Briejer *et al.*, 1992; Briejer *et al.*, 1994b). Furthermore it was shown, that the recently identified inhibitory neurotransmitter nitric oxide (NO) (for review, see Stark & Szurszewski 1992) is involved in the 5-HT-induced relaxations (Briejer *et al.*, 1992). However, the relaxations to 5-HT are only partially sensitive to N^G-nitro-

L-arginine (L-NNA), an inhibitor of the NO synthesis. Furthermore, 5-HT could release a transmitter which in turn causes the generation of NO. Though it is now generally accepted that NO is an important and widespread inhibitory neurotransmitter in the gut (Stark & Szurszewski, 1992), vasoactive intestinal polypeptide (VIP) and adenosine 5'-triphosphate (ATP) are also still on the list of candidates (Burnstock, 1972; Goyal *et al.*, 1980; Kennedy, 1990; Crist *et al.*, 1992).

In the guinea-pig taenia caeci, which is located anatomically proximal to the proximal colon, a role for ATP as an inhibitory neurotransmitter has been suggested (Burnstock, 1972; Maas *et al.*, 1980). ATP generally causes relaxation of gastrointestinal smooth muscle through stimulation of postsynaptic P_{2y} purinoceptors (see: Kennedy, 1990). Several pharmacological tools are available to inhibit these purinoceptor-mediated responses. The trypanocide suramin and the anthraquinone-sulphonic acid derivative dye reactive blue 2 have been used as specific purinoceptor antagonists (Burnstock & Warland, 1987; Den Hartog *et al.*, 1989; Hoyle *et al.*, 1990; see for review: Kennedy, 1990). α,β-methylene-ATP (α,β-Me-ATP), an ATP analogue that is resistant to enzymatic degradation, is generally used to desensitize the P_{2x} purinoceptors, mediating contraction in smooth muscle preparations; still, desensitization of P_{2y} purinoceptors inducing relaxation has been obtained with α,β-Me-ATP in some parts of the gastro-intestinal tract (Kennedy, 1990). The polypeptide bee venom apamin has been used in several guinea-pig intestinal preparations as an effective antagonist of inhibitory responses to externally applied ATP (Shuba & Vladimirova, 1980; Maas *et al.*, 1980; Costa *et al.*, 1986).

Vasoactive intestinal (poly)peptide (VIP) is a neurotransmitter of the enteric nervous system (Goyal *et al.*, 1980; Grider *et al.*, 1985). Its presence in enteric neurones of the guinea-pig has been demonstrated with immunohistochemical methods (Costa & Furness, 1983; Probert *et al.*, 1983). In most gastrointestinal smooth muscle preparations, VIP causes relaxations (Goyal *et al.*, 1980; Grider *et al.*, 1985) through a direct action on the smooth muscle, though in a minority of tissues neurogenic contractions to VIP have been observed, e.g. in the guinea-pig ileum (Katsoulis *et al.*, 1993).

The objectives of the current study were, to assess whether ATP and/or VIP, either as an intermediate or end transmitter, could also be involved in the 5-HT-induced relaxations of the guinea-pig colon.

Materials and methods

Dunkin-Hartley guinea-pigs of both sexes, weighing 400-600 g, were killed by decapitation. The proximal colon was removed, and the luminal contents were washed out with De Jalon solution (mM: KCl 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, NaCl 155, glucose 2.78). The mesentery was carefully removed. Starting at the proximal end, about 1 cm distal from the caecum, the colon was divided into four segments of circa 2.5 cm. These intact segments were individually mounted vertically for isotonic measurement into a tissue bath containing 20 ml De Jalon solution. This solution was kept at 37.5 °C and gassed with 95% O_2, 5 % CO_2. The strips were subjected to a preload of 2 g and allowed to stabilize for half an hour. Ketanserin (0.3 μM) and tropisetron (ICS 205-930, 3 μM) were continuously present in the De Jalon solution during all experiments to block contractile responses mediated by 5-HT_2, 5-HT_3 and 5-HT_4 receptors. After stabilization, the longitudinal muscle was challenged with 0.3 μM methacholine. After washing and 10 min stabilization, the procedure was repeated in order to stabilize the response of the tissue.

Protocols

For the construction of concentration-response curves, agonists and antagonists were applied directly to the tissue bath (added volume ≤ 3 % of tissue bath volume). Methacholine (0.3 μM) was added to the bath in order to precontract the muscle.

5-HT. Since pilot experiments had revealed that relaxations to 5-HT are subjected to tachyphylaxis, non-cumulative concentration-response curves were established, beginning 10 min after the administration of methacholine. The size of the relaxation was measured as the maximum relaxation that was induced during an incubation period of approximately 2 min (at high concentrations in some cases up to 4 min). Then, 5-HT and MeCh were washed out by replacing the bathing fluid twice. Stabilization of 10 min was allowed. This 20 min dosing cycle was repeated applying the next concentration of 5-HT. The response to repetitive administration of methacholine was stable throughout the experiment. Each segment was first challenged with 10 μM 5-HT in the absence of an antagonist (ketanserin and tropisetron present). After washout this cycle was repeated, applying again 10 μM 5-HT. The relaxation to this latter administration of 5-HT was taken as 100 % relaxation. Consecutively, antagonists were administered (and their intrinsic effects, if any, were assessed), and were left with the prepara-

tions for 15 min. Then, non-cumulative concentration-response curves were established, applying the above described 20 min dosing cycle. Antagonists were re-added directly after each washout. Of 4 segments that were taken from each animal, one strip was utilized as a control. The antagonists had no effect on the methacholine-induced contractions. The concentrations of 5-HT were applied in ascending order. However, when α-chymotrypsin and trypsin were tested against 5-HT-induced relaxations, 5-HT was applied in descending order, for the peptidases tended to increase the basal length of some of the preparations after several hours. When an ascending concentration administration order is applied, the largest relaxations are in the end of the experiment. It is likely that these large relaxations are more affected by the increase in length of the strips due to the proteases than would be small relaxations. To avoid this, a descending order was chosen.

ATP. A protocol identical to that of 5-HT was applied for ATP. The only difference was, that prior to the establishment of the concentration-response curves, a relaxation to 5-HT 10 μM was established. All consecutive relaxations were expressed as a percentage of this relaxation to 5-HT. The optimum antagonist concentration of suramin to inhibit ATP 3 μM-induced relaxations was determined, by preincubation of the strips with increasing concentrations of suramin or solvent, in a similar protocol as described above.

NO. In an identical 20 min dosing cycle as described for 5-HT, a relaxation to 1 μM NO was induced twice; for each strip, the relaxation to this second relaxation was taken as 100 %. Then, after washout and a 10 min stabilization period, antagonists or vehicle (= control) were added, and a 10 min incubation period was allowed. Consecutively, methacholine was administered to induce a contraction, and after another 10 min, NO (1 μM) was administered as well.

Nitroglycerin, isoprenaline and VIP. For the construction of concentration-response curves to nitroglycerin, isoprenaline and VIP, the compounds were added in a cumulative fashion, 10 min after precontraction to methacholine-addition (0.3 μM). After washing and stabilization for 30 min (washing every 10 min), a second concentration-response curve was established in the presence of an antagonist. The relaxations to nitroglycerin, isoprenaline and VIP were reproducible in time. The responses induced by nitroglycerin, isoprenaline and VIP were expressed relative to the response to respectively nitroglycerin 10 μM, isoprenaline 3 μM or VIP 0.3 μM of the first series for each segment.

Desensitization to α,β-Me-ATP. First, the strips were precontracted with methacholine 0.3 μM, and after 10 min, the relaxation to 5-HT 10 μM was established. All further relaxations in this experiment were expressed as a percentage of this relaxation to 5-HT. Consecutively, after washout and 10 min stabilization, the strips were again precontracted with methacholine, and after 10 min a relaxation to ATP, was established. After washout and 10 min stabilization, α,β-Me-ATP 100 μM was administered. Again 10 min later, the precontraction and relaxation cycle with ATP was repeated.

Chemicals

Methacholine HCl (E. Merck, Germany), nitroglycerin (Federa, Belgium), suramin sodium (Rhone-Poulenc, France), 5-HT creatinine sulphate, tetrodotoxin, apamin, vasoactive intestinal polypeptide (VIP) (Serva, Germany), isoprenaline tartrate, N^G-nitro-L-arginine, reactive blue 2 (procion blue HB, Cibacron blue F3GA), ATP disodium salt (Janssen Chimica, Belgium), phosphoramidon, bovine trypsin (type III; specific activity: 10700 BAEE units.mg^{-1}), bovine α-chymotrypsin (type II; specific activity: 40-60 BTEE units.mg^{-1}), VIP_{10-28} (acetate salt), [p-Cl-D-Phe^6,Leu^{17}]VIP, α,β-methylene-ATP lithium salt (Sigma, Belgium) were added to the tissue bath solution in volumes ≤ 3% of the bath volumes. Ketanserin tartrate and tropisetron HCl (ICS 205-930; Janssen Research Foundation, Belgium) were continuously present in the De Jalon solution. All compounds were dissolved in distilled water. The stock solution for isoprenaline and 5-HT contained 0.25 μM ascorbic acid. This vehicle had no effect on the strips. Glyceryl trinitrate was further diluted with distilled water from a stock solution of 1 % in ethanol. The stock solutions of apamin, TTX, VIP, VIP_{10-28}, [p-Cl-D-Phe^6,Leu^{17}]VIP and phosphoramidon were prepared in advance and kept frozen (-30 °C). The solutions of the peptides mentioned above, as well as those of phosphoramidon, TTX and α,β-Me-ATP were kept on ice during the experiments. Phosphoramidon and trypsin had no effect on the length of the strips; N^G-nitro-L-arginine, apamin, suramin and tetrodotoxin induced a transient shortening of the strips, but the level of precontraction by methacholine was unaffected. NO solution was kindly provided by Drs. J. De Man and G. Boeckxstaens. The solution was prepared as described elsewhere (Boeckxstaens *et al.*, 1991), which yields a saturated solution of about 2 mM (Feelish, 1991). The NO solution was protected from air and kept on ice during the experiment. Addition of trypsin immediately caused complex effects on the strips (relaxations as well as contractions) which tended to fade within several min, but the level of precontraction was not affected. As stated before, trypsin and α-chymotrypsin

tended to increase the length of some strips after several hours, probably caused by their digestive action on the integrity of the tissue.

Statistical analysis

For graphical representation means ± standard error of the means (S.E.) were calculated. Differences between mean values were tested with one way analysis of variance using the Scheffé *F*-test (comparison treatment vs. treatment) or Dunnett's *t*-test (treatment vs. control) (Wallenstein *et al.*, 1980). The level of significance was set at $P < 0.05$.

Results

5-HT induced concentration-dependent relaxations (Figure 6.1, *upper panel*) that were fast in onset. Maximum relaxations were observed at 30 μM 5-HT. In about 1 out of 3 preparations, the relaxations to higher concentrations of 5-HT were multiphasic, i.e. an initial quick relaxation followed by a small contraction and consecutively a more sustained relaxation (Figure 6.1, *middle panel*). The concentration-response curve to 5-HT appeared non-sigmoidal (Figure 6.2). ATP induced concentration-dependent relaxations (Figure 6.1, *middle panel*) that were fast in onset. The ATP-induced relaxations were short lasting, and were mostly followed by a second, more sustained relaxation. At higher concentrations of ATP, this slow, more sustained second relaxation exceeded the first, fast relaxation (Figure 6.1, *middle panel*). The concentration-response curve to ATP is shown in Figure 6.2. As relaxations to 10 μM ATP approximately equalled maximum relaxations to 5-HT (Figure 6.2), higher concentrations of ATP were not tested for the construction of the ATP concentration-relaxation curve. Both in case of 5-HT and ATP, the maximum relaxation to each agonist concentration was measured for quantification of the responses, irrespective to whether this maximum relaxation occurred in the first, fast relaxation or the second, more sustained relaxation. VIP induced slowly developing concentration-dependent relaxations from 3 nM onwards, which did not fade (Figure 6.1, *lower panel*).

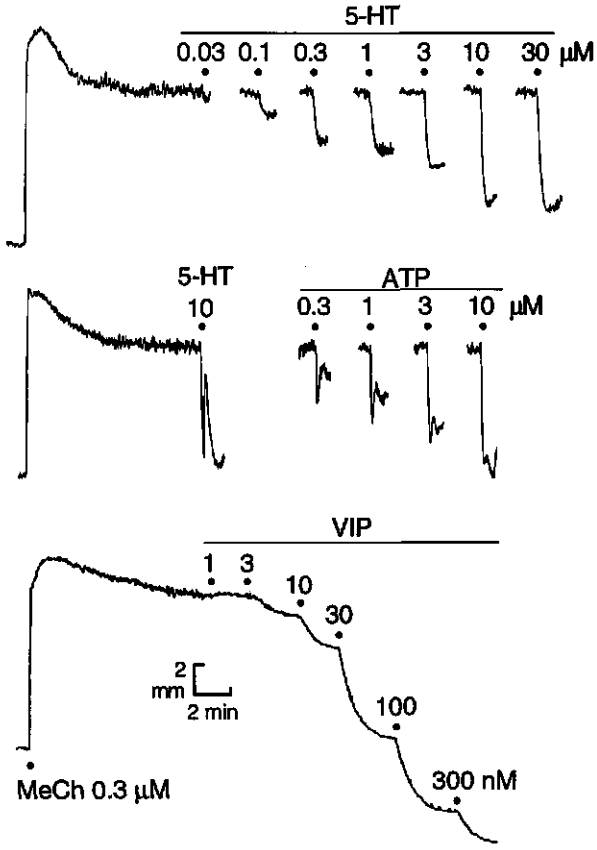


Figure 6.1 Representative tracings of the relaxations to 5-hydroxytryptamine (5-HT; *upper tracing*), adenosine triphosphate (ATP; *middle tracing*) and vasoactive intestinal polypeptide (VIP; *lower tracing*). The preparations were precontracted with methacholine, whereafter 5-HT, ATP or VIP were added. Antagonists were continuously present to block contractile responses elicited *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors.

At low concentrations (3 nM), some strips responded after a short lag time (maximally about 0.5 min), but at higher concentrations no lag time was observed. The concentration-response curve of the relaxations to VIP was sigmoidal, with a near maximum effect at 0.3 µM VIP. Maximum relaxations to 5-HT were on average only about half in magnitude of that caused by VIP. VIP inhibited the spontaneous movements of the strips. Isoprenaline induced slowly developing relaxations, whereas those to nitroglycerin were fast in onset. The

maximum relaxations to isoprenaline and nitroglycerin were on average about twice as large as those to 5-HT.

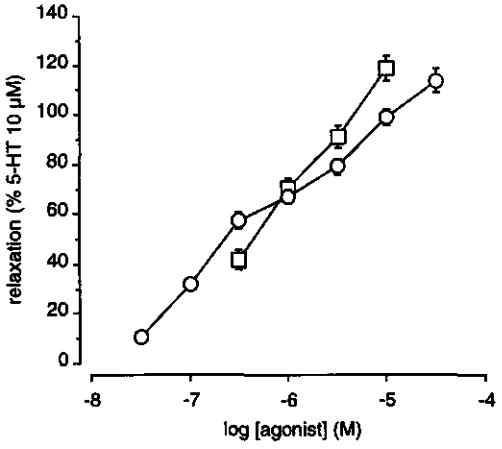


Figure 6.2 The concentration-response curves to ATP (□) and 5-HT (○) (n = 14 - 29; means ± S.E.). The relaxations to ATP and 5-HT were expressed as a percentage of relaxations induced by 5-HT 10 µM.

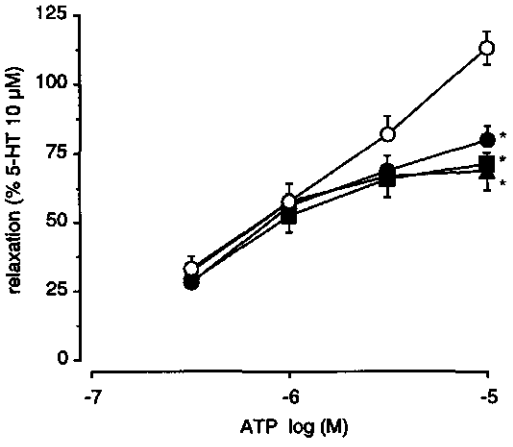


Figure 6.3 Concentration-response curves to adenosine triphosphate (ATP) in the absence of drugs (○) or in the presence of tetrodotoxin 0.3 µM (■), N^G-nitro-L-arginine 100 µM (●), or both tetrodotoxin and N^G-nitro-L-arginine (▲). Values (means ± S.E.; n = 6) that are significantly different from control ($P < 0.05$) are marked with an asterisk. The relaxations to ATP were expressed as a percentage of relaxations to 5-hydroxytryptamine (5-HT) 10 µM.

Tetrodotoxin (TTX) and N^G-nitro-L-arginine (L-NNA)

The neuronal conductance blocker TTX (0.3 μM) did not affect the responses to ATP up to 3 μM, but at 10 μM ATP, it significantly inhibited the relaxations due to ATP (Figure 6.3). The NO synthase inhibitor L-NNA (100 μM) had a similar effect on the relaxations to ATP, as did the combination treatment with TTX and L-NNA (Figure 6.3). TTX (0.3 μM) did not significantly affect the concentration-response curve to VIP (n = 4), nor did L-NNA (100 μM; n = 4) (not shown).

Suramin

The putative P_2-purinoceptor antagonist suramin, at a concentration ≥ 30 μM, inhibited relaxations to ATP 3 μM significantly, while the inhibition was maximal at 300 μM suramin (n = 8; not shown). Suramin (300 μM) induced transient contractions (11.0 ± 3.8 % of 5-HT-induced relaxations; n = 6) and it had no significant effect on the magnitude of the methacholine-induced precontractions. Suramin strongly inhibited the relaxations to ATP up to 3 μM (Figure 6.4, *upper panel*). Surprisingly, the concentration-relaxation curve to VIP was also strongly depressed (Figure 6.4, *middle panel*). Suramin (300 μM) did not significantly influence the concentration-relaxation curves to isoprenaline and nitroglycerin (n = 4; not shown). Relaxations to exogenous NO (ca. 1 μM) were also not significantly inhibited by suramin (300 μM; n = 8, not shown). Suramin inhibited the relaxations to 5-HT in the low concentration range (up to 3 μM), but the relaxations to higher concentrations of 5-HT were more strongly inhibited, rendering a monophasic sigmoidal concentration-response curve (Figure 6.3, *lower panel*; n = 8). As observed before (Briejer *et al.*, 1992), L-NNA (100 μM) strongly inhibited the relaxations to 5-HT. In the presence of L-NNA (100 μM), suramin (300 μM) could not significantly inhibit the relaxations to 5-HT any further (Figure 6.4, *lower panel*; n = 8).

Apamin

The Ca^{2+}-dependent K^+-channel blocker apamin (30 and 600 nM) induced short-lasting contractions (resp. 33.7 ± 5.4 % and 51.7 ± 6.8 % of 5-HT-induced relaxations, n = 6), and it had no effect on the magnitude of the methacholine-induced precontractions. Apamin 30 nM strongly inhibited the relaxations to ATP (Figure 6.5, *upper panel*; n = 4); at 600 nM, apamin could not inhibit the relaxations to ATP any further (n = 4; not shown), which indicates that 30 nM is a fully effective concentration. Up to 3 μM ATP (i.e. relaxations which are in magnitude comparable to maximum relaxations to 5-HT), apamin inhibited the

ATP relaxations by 70-100 % (Figure 6.4, *upper panel*). Apamin (30 nM) inhibited relaxations to VIP only significantly at 30 nM VIP (Figure 6.5, *middle panel*), while it depressed the concentration-response curve to isoprenaline by about 10-20 % (n = 4; not shown). The relaxations to nitroglycerin were not significantly affected by the presence of apamin 30 nM (n = 4; not shown). Apamin 600 nM did not significantly inhibit the relaxations to NO (1 μM; n = 8, not shown; apamin 30 nM was not tested against NO). Apamin (30 nM) inhibited the relaxations to 5-HT, the inhibition being more prominent at higher concentrations of 5-HT (Figure 6.4, *lower panel*). In the presence of L-NNA (100 μM), apamin (30 nM) only significantly inhibited relaxations to 5-HT 30 μM.

Miscellaneous substances

The neutral endopeptidase inhibitor phosphoramidon (1 μM) had no significant effect on the relaxations to VIP (n = 6; not shown), nor did it affect the concentration-response curve to 5-HT (n = 6; not shown). The putative VIP receptor antagonists [p-Cl-D-Phe^{6},Leu^{17}]VIP (1 μM) and VIP$_{10-28}$ (1 and 3 μM) induced a transient contraction of respectively 13.5 ± 6.3 %, 29.8 ± 3.3 % and 58.7 ± 8.9 % of VIP (0.3 μM)-induced relaxations (n = 4). By the time that VIP was administered, the effects of the VIP receptor antagonists had disappeared. Neither of these putative VIP receptor antagonists affected the relaxations to exogenously applied VIP, yielding concentration-response curves that coincided with controls (n = 4; not shown). The peptidases α-chymotrypsin and trypsin had no effect against isoprenaline-induced relaxations at a concentration of respectively 0.015 mg.ml^{-1} and 0.005 mg.ml^{-1} (n = 5; not shown). However, higher concentrations of the peptidases (0.010 mg.ml^{-1} and 0.030 mg.ml^{-1} respectively) caused a significant decrease of both the methacholine-induced precontractions and the relaxations to isoprenaline, and therefore the former concentrations were used to test against VIP. α-Chymotrypsin (0.015 mg.ml^{-1}) as well as trypsin (0.005 mg.ml^{-1}) abolished the relaxations to VIP up to 0.03 μM, and strongly inhibited them up to 0.3 μM VIP (n = 4; Figure 6.6). Neither α-chymotrypsin (0.015 mg.ml^{-1}), nor trypsin (0.005 mg.ml^{-1}) significantly ($P > 0.05$) affected the concentration-response curve to 5-HT (n = 6; not shown).

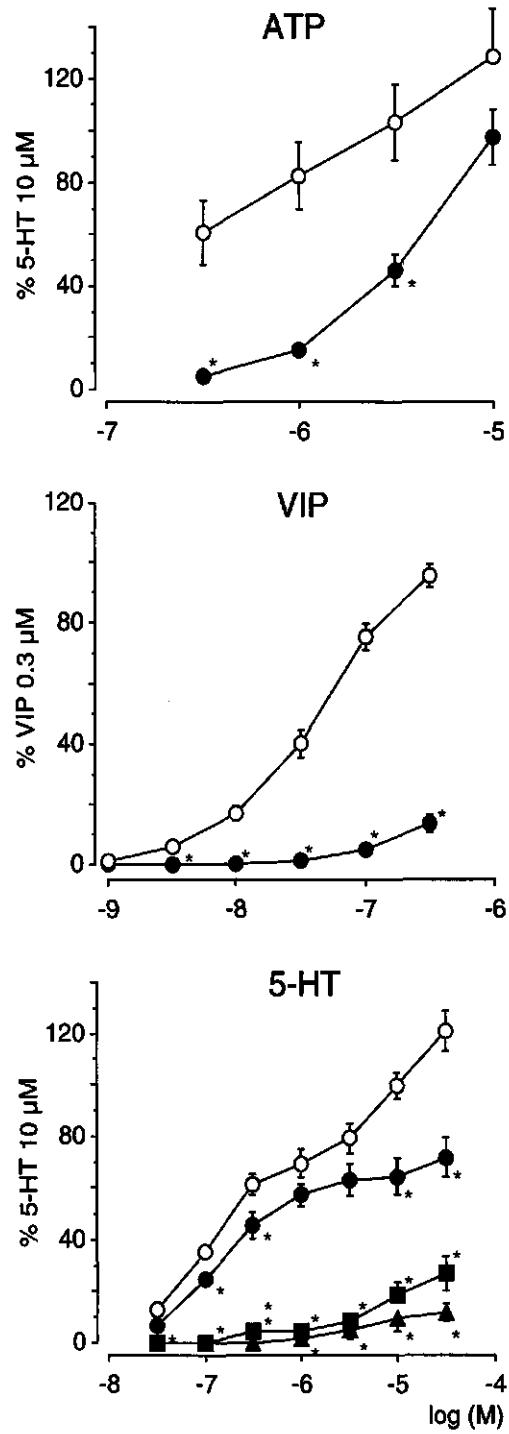


Figure 6.4 The *upper panel* shows a concentration-response curve to adenosine triphosphate (ATP) in the absence (O) and presence (●) of suramin 300 µM. Suramin 300 µM (●) also strongly inhibited the relaxations to vasoactive intestinal polypeptide (VIP) (O = control; *middle panel*). The *lower panel* shows concentration-response curves to 5-hydroxytryptamine (5-HT): control (O) or in the presence of suramin 300 µM (●), N^G-nitro-L-arginine (L-NNA) 100 µM (■), or both suramin and L-NNA (▲). Values (means ± S.E.; n = 4 - 8) that are significantly different from control ($P < 0.05$) are marked with an asterisk. The relaxations to ATP were expressed as a percentage of relaxations to 10 µM 5-HT, whereas the relaxations to VIP were expressed as a percentage of the relaxations to 0.3 µM VIP.

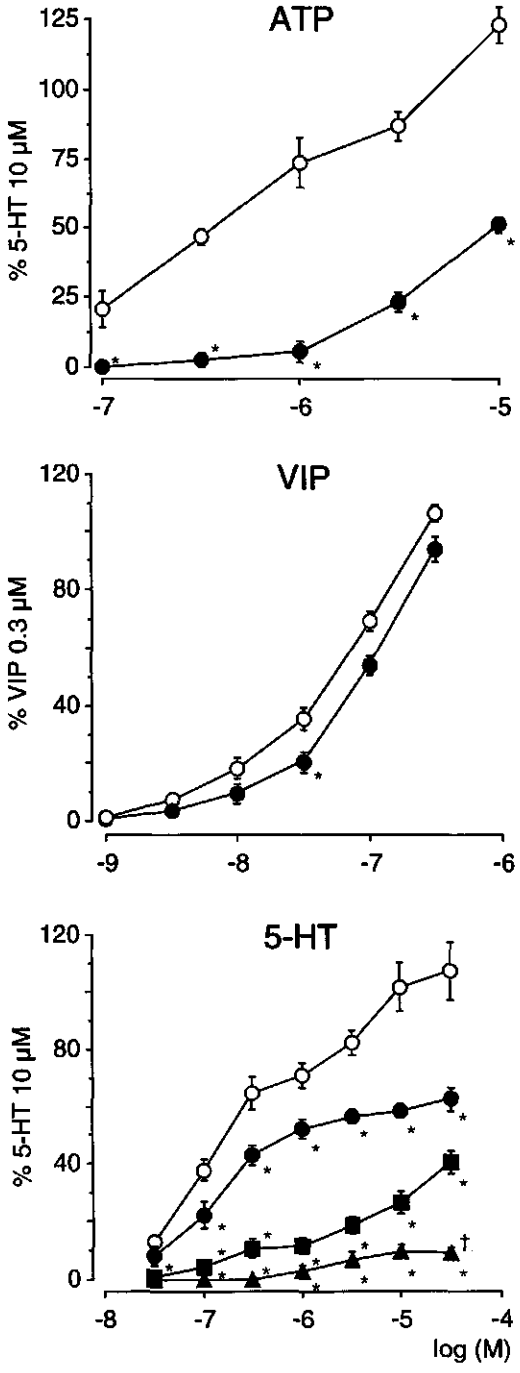


Figure 6.5 The *upper panel* shows a concentration-response curve to adenosine triphosphate (ATP) in the absence (O) and presence (●) of apamin 30 nM. Apamin 30 nM (●) inhibited the relaxations to vasoactive intestinal peptide (VIP) (O = control) only at 30 nM VIP (*middle panel*). The *lower panel* shows concentration-response curves to 5-hydroxytryptamine (5-HT): control (O) or in the presence of apamin 30 nM (●), N^G-nitro-L-arginine (L-NNA) 100 µM (■), or both apamin and L-NNA (▲). Values (means ± S.E.; n = 4-6) that are significantly different from control ($P < 0.05$) are marked with an asterisk; significant difference from treatment with L-NNA is denoted with †. The relaxations to ATP were expressed as a percentage of relaxations to 10 µM 5-HT, whereas the relaxations to VIP were expressed as a percentage of the relaxations to 0.3 µM VIP.

α,β-Me-ATP

The metabolic degradation-resistant ATP analogue α,β-Me-ATP 100 μM induced a lasting relaxation (139 ± 17 % of 5-HT-induced relaxations, 8 strips), without any sign of fading. When methacholine 0.3 μM was subsequently added to precontract the muscle, it induced a transient contraction (normally a tonic contraction), followed by phasic contractions in 7 out of 8 strips (normally a stable line). When after 10 min ATP 3 μM was added as well, it still induced relaxations (64 ± 6 % of the ATP-induced relaxations before treatment, n = 4). Another experiment revealed, that even after 2 h and a second administration of α,β-Me-ATP after 1 h, consecutive relaxations to ATP were not at all desensitized by use of α,β-Me-ATP (n = 4, not shown). From these results it is clear, that under the present experimental conditions, it is not possible to desensitize the inhibitory responses to ATP. Therefore, no further experiments were performed with this compound.

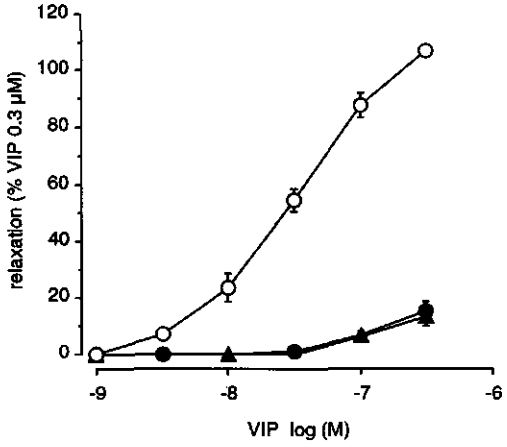


Figure 6.6 Concentration-response curves to vasoactive intestinal polypeptide (VIP): control (O), or in the presence of α-chymotrypsin (●) (0.015 mg.ml^{-1}) or trypsin (▲) (0.005 mg.ml^{-1}). The relaxations to VIP were expressed as a percentage of relaxations to VIP (0.3 μM) in the absence of a peptidase. Values represent the means ± SE of 4 experiments.

Reactive blue 2

The putative P_{2Y}-purinoceptor antagonist reactive blue 2, in concentrations from 10 μM onwards, significantly inhibited the relaxations to isoprenaline,

depressing the concentration-response curve (10 μM: 30 % depression of maximum; 30 μM: 45 % depression). Reactive blue 3 μM did not affect the relaxations to ATP. The contractions to methacholine were also inhibited (up to 30 %) by reactive blue 2 in the tested concentrations; these effects seemed to increase with time. Hence, in our preparation, reactive blue 2 could not be used as an antagonist to study ATP-mediated responses.

Discussion

The results of the current study suggest that, in association with NO (Briejer *et al.*, 1992; current results) ATP might be involved in the mediation of 5-HT-induced relaxations in the guinea-pig proximal colon longitudinal muscle. Evidence for the involvement of VIP or another peptide was not obtained.

ATP caused the preparations to relax, a concentration of 3 μM being about as effective as 5-HT 10 μM. A slightly higher concentration of ATP induced relaxations that were partially sensitive to the blocker of NO synthase, L-NNA, and the neurotoxin TTX; the combined treatment caused no additional inhibition. VIP-induced relaxations were not inhibited by either TTX or L-NNA, suggesting an action directly on the smooth muscle. These observations suggest that VIP and ATP at lower concentrations act mainly directly on the smooth muscle, although at higher concentrations ATP may also release some NO from a neuronal source.

Suramin inhibited the relaxations to exogenously applied ATP. In contrast, it did not affect the relaxations to either isoprenaline, which acts by stimulation of post-junctional β-adrenoceptors (Broadly & Grassby 1985), or nitroglycerin, which generates NO (NO-synthase independent) (Feelish, 1991). Relaxations induced by exogenous NO were also not affected by suramin. Many other studies have demonstrated the antagonist specificity of suramin for P_2 purinoceptor-mediated responses (Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990; see also: Kennedy, 1990). Relaxations to ATP, that were in magnitude comparable to submaximal relaxations to 5-HT, were virtually abolished by suramin. Thus, if 5-HT were to act solely through the release of ATP, its relaxations should be potently inhibited by suramin which is not the case. It has been shown previously, that the greater part of the relaxations to 5-HT is mediated by NO as a final transmitter (Briejer *et al.*, 1992). Exogenous NO mimicked the relaxations to 5-HT, and the relaxations to 5-HT, but not NO, were sensitive to inhibition by L-NNA. Hence, the observations with L-NNA in this study confirm this conclusion. Both NO and ATP relax the smooth muscle mainly by a direct action (Briejer *et al.*, 1992; current results).

As L-NNA inhibits most of the relaxation to 5-HT, if ATP is involved as well as NO, the concentration of endogenously released ATP is likely to be low. Therefore, relaxations to endogenous concentrations of ATP should be abolished by suramin. Indeed, suramin did partially inhibit the relaxations to 5-HT, especially at higher concentrations of 5-HT. At these higher concentrations, L-NNA could not completely block the relaxations to 5-HT. However, in the presence of L-NNA, suramin did not significantly further inhibit the relaxations. This might indicate that the endogenously released NO acts synergistically with ATP in relaxing the smooth muscle.

Surprisingly, it was found that suramin is also an effective inhibitor of VIP-induced relaxations in this preparation. This observation undermines the contention that suramin is a specific purinoceptor antagonist. Furthermore, the observed inhibition by suramin against 5-HT-induced relaxations, could also suggest that VIP instead of or next to ATP is involved.

A second set of observations strengthens the ATP hypothesis. The ATP-induced relaxations, but not those to VIP, were potently inhibited by apamin. Shuba and Vladimirova (1980) discovered that apamin abolishes the hyperpolarizing action of externally applied ATP in guinea-pig intestinal preparations. Apamin was also found to inhibit the relaxation and the inhibitory junction potentials that are evoked when non-adrenergic non-cholinergic nerves are electrically stimulated and which have been associated with the facilitation of purinergic transmission (Shuba & Vladimirova 1980). These observations might well be explained by the Ca^{2+}-dependent K^+-channel blocking properties of apamin (Banks *et al.*, 1979; Maas *et al.*, 1980). However, the specificity of apamin has been discussed, for it has been shown that it does not only inhibit hyperpolarizations and inhibitory junction potentials to ATP. NO and noradrenaline or noradrenergic nerve stimulation also evoke similar inhibitory responses, that in some tissues are inhibited by apamin (Banks *et al.*, 1979; Ward *et al.*, 1992). We have previously shown, that noradrenaline is not involved in the 5-HT-induced relaxations (Briejer *et al.*, 1992). Furthermore, it is known that not all Ca^{2+}-dependent K^+-channels are sensitive to apamin (Jury *et al.*, 1985; Ward *et al.*, 1992), which suggests the existence of several subtypes of these K^+-channels. In our preparation, apamin only slightly inhibited the relaxations to isoprenaline, but not those to nitroglycerin, NO or VIP. In other studies it was also reported that, in similar preparations, apamin (0.5 μM) slightly inhibited the relaxations to isoprenaline and VIP (Costa *et al.*, 1986). Since, in contrast, apamin potently inhibited relaxations induced by ATP (current results) and α,β-Me-ATP (Costa *et al.*, 1986), it seems that apamin is, in this preparation, a relatively specific antago-

nist of the relaxations to ATP. We therefore tested it against the relaxations to 5-HT, and found that apamin caused marked inhibition, especially in the higher 5-HT concentration range. These observations confirm the above results, suggesting a role for ATP, but not VIP, in the 5-HT-induced relaxations.

To further strengthen our hypothesis, we attempted to seek for more pharmacological tools. Reactive blue 2, previously described as a P_2 purinoceptor antagonist (slightly selective for the P_{2y} purinoceptor) (Burnstock & Warland, 1987; Crist *et al.*, 1992), caused many non-specific effects in our preparation. At concentrations necessary to inhibit ATP-induced relaxations (in gastrointestinal preparations ≥ 30 μM: Lefebvre & Burnstock 1990), it inhibited relaxations to isoprenaline as well. It was therefore not possible to test reactive blue 2 against 5-HT-induced relaxations. Other workers have also observed these non-specific (time-dependent) effects, and it has been suggested, that the impurity of commercially available reactive blue 2 (60 % pure) may well cause these problems (Burnstock & Warland, 1987).

It was also investigated whether it was possible to desensitize the purinoceptor responsible for the relaxations to ATP. Since ATP is a metabolically and chemically rather unstable agent, α,β-Me-ATP, resistant to metabolic degradation, was used. α,β-Me-ATP is usually more effective than ATP as a desensitizing agent (Kennedy, 1990). However, α,β-Me-ATP induced non-fading relaxations, and the relaxations to exogenous ATP applied subsequently did not appear inhibited. From the above discussed observations it thus seems that the P_2-purinoceptors under the applied experimental conditions are not prone to desensitization. It is known, though not understood, that in some parts of the gastrointestinal tract, repeated administration of ATP or α,β-Me-ATP does not lead to desensitization of the inhibitory (P_{2y} purinoceptor-mediated) responses, whereas in other parts it does (Kennedy, 1990). Based on the observations discussed above, no further experiments with α,β-Me-ATP were therefore undertaken.

Neural endopeptidase EC 3.4.24.11 (NEP), an enzyme which degrades endogenous peptide transmitters, has been shown to be present in the guinea-pig gastrointestinal tract (Nau *et al.*, 1986; Gu *et al.*, 1993). Phosphoramidon, a potent inhibitor of NEP (IC_{50} ~ 5 nM; Gu *et al.* 1993), has been reported to potentiate the inhibitory response to exogenous VIP in guinea-pig trachea (Rhoden & Barnes 1989). In our preparation, phosphoramidon did not affect the relaxations to exogenous VIP or to 5-HT. These results either suggest that NEP is not present in a concentration that is high enough to significantly influence the concentration

of VIP at its site of action, or that VIP (or another NEP-degradable peptide) is not involved in the relaxations to 5-HT.

To date, a potent and selective VIP receptor antagonist is still not available. The antagonists that have been claimed to possess VIP antagonistic activity are analogues of VIP or GRF (which structurally is a member of the VIP peptide family), and need to be applied at micromolar concentrations (see for review: Presti & Gardner, 1993). [p-Cl-D-Phe^6,Leu^{17}]VIP (IC_{50} at VIP receptor ~ 3 μM, Pandol *et al.*, 1986) was tested at a concentration of 1 μM and the VIP fragment VIP_{10-28} (IC_{50} ~ 1 μM, Turner *et al.*, 1986) at 1 μM and also at 3 μM. At the tested concentrations, however, they did not have any effect on the relaxations to exogenous VIP. Other studies have also reported on the ineffectiveness of these putative VIP receptor antagonists against responses to VIP, even up to a concentration of 30 μM (D'Amato *et al.*, 1988; De Beurme & Lefebvre, 1988; Ellis & Farmer, 1989a). Thus, if these antagonists were to have any antagonistic effect in our tissues, some inhibition was to be expected at the applied concentractions. For example, in guinea-pig airway tissue, 1 μM [p-Cl-D-Phe^6,Leu^{17}]VIP significantly inhibited relaxations to exogenous VIP (Venugopalan *et al.*, 1990). Interestingly, both [p-Cl-D-Phe^6,Leu^{17}]VIP and VIP_{10-28} induced a contraction in our preparation. In a guinea-pig ileum preparation, it has been shown that VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) evoke a neurogenic contraction (Katsoulis *et al.*, 1993). These observations and the ineffectiveness of the antagonists to inhibit VIP-induced relaxations in our preparation might be explained in terms of regional differences of VIP receptors and different subtypes of VIP/PACAP receptors. Since the putative VIP antagonists were ineffective against VIP itself in our preparations, they were not tested against 5-HT-induced relaxations to investigate the involvement of VIP.

Peptidases like trypsin and α-chymotrypsin have been successfully applied to investigate peptidergic involvement in relaxatory responses in guinea-pig, human and rat tissues (De Beurme & Lefebvre, 1987; Ellis & Farmer 1989b; Belvisi *et al.*, 1993). It cannot be excluded that the peptidases did not reach the site of action of VIP because of their large molecular size. However, the specific inhibition by α-chymotrypsin and trypsin of electrical field stimulation-induced peptidergic responses, suggests that the peptidases do penetrate tissues (De Beurme & Lefebvre, 1987; D'Amato *et al.*, 1988; Ellis & Farmer, 1989b; Belvisi *et al.*, 1993). In our preparation, trypsin and α-chymotrypsin almost abolished relaxations to externally applied VIP, while leaving the relaxations to isoprenaline unaffected. Hence, trypsin and α-chymotrypsin are suitable to investigate the involvement of VIP in 5-HT-induced relaxations in our prepara-

tion. However, neither trypsin nor α-chymotrypsin affected the relaxations to 5-HT in any respect. These results suggest that VIP is not involved in the 5-HT-induced relaxations The action of trypsin and α-chymotrypsin against relaxatory peptides other than VIP has not been investigated in our preparation.

Summarizing, the results as presented here favour the involvement of a second transmitter next to NO in 5-HT-induced relaxations of the guinea-pig colon. Suramin inhibited the relaxations to 5-HT, ATP and VIP, but apamin was only effective against 5-HT and ATP, which suggest ATP is involved. Furthermore, the lack of effect of α-chymotrypsin and trypsin against 5-HT-induced relaxations argues against involvement of a peptide. As relaxations to ATP (and to VIP and NO: current results and Briejer *et al.* 1992) were shown to be (largely) caused *via* a direct effect on the smooth muscle, not involving NO synthesis, it is suggested that NO and ATP are both released in parallel and act in a synergistic way. The biphasic concentration-response curve to 5-HT could suggest that even two 5-HT receptor types are involved, a high affinity 5-HT receptor mediating NO generation and a low affinity 5-HT receptor mediating ATP release. Direct and indirect evidence for a two receptor system has also been obtained with the use of several 5-HT receptor antagonists (Briejer *et al.*, 1994a; b).

It can be concluded that on the guinea-pig colon longitudinal muscle, VIP and ATP induce relaxation *via* a direct effect of the smooth muscle, not involving NO. 5-HT-induced relaxations are mediated by NO as well as by a substance which is sensitive to inhibition by suramin and apamin. It is suggested that this substance is ATP and not a peptide.

Acknowledgements

We thank Drs. J. De Man and G. Boeckxstaens for the preparation and kind donation of the NO solution.

References

Banks BEC, Brown C, Burgess GM, Burnstock G, Claret M, Cocks TM, Jenkinson DH (1979) Apamin blocks certain neurotransmitter-induced increases in potassium permeability. *Nature* 282: 415-417.

Belvisi MG, Miura M, Stretton D, Barnes PJ (1993) Endogenous vasoactive intestinal peptide and nitric oxide modulate cholinergic neurotransmission in guinea-pig trachea. *Eur J Pharmacol* 231: 97-102.

Boeckxstaens GE. Pelckmans PA, Bult H, De Man JG, Herman AG, Van Maercke YM (1991). Evidence for nitric oxide as mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut. *Br J Pharmacol* 102: 434-438.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1992) Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*. *Br J Pharmacol* 107: 756-761.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1993) Pharmacological characterization of 5-hydroxytryptamine receptor types in the guinea-pig proximal colon. *J Gastroint Motil* 5: 239-246.

Briejer MR, Veen GJ, Akkermans LMA, Schuurkes JAJ (1994a) Cisapride facilitates inhibitory purinergic transmission in the guinea pig colon. *Gastroenterology* 106: A471.

Briejer MR, Akkermans LMA, Schuurkes JAJ (1994b) Novel 5-HT receptor type mediating neurogenic relaxation of guinea pig colon. In: *Proceedings of the Third IUPHAR Satellite Meeting of Serotonin* (Huston JP, Ed.), Elsevier: in press.

Broadly KJ, Grassby PF (1985) Alpha- and beta-adrenoceptor-mediated responses of the guinea-pig ileum and the effects of neuronal uptake inhibition. *Naunyn-Schmiedeberg's Arch Pharmacol* 333: 316-323.

Burnstock G (1972) Purinergic nerves. *Pharmacol Rev* 24: 509-581.

Burnstock G, Warland JJI (1987) P_2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated *via* the P_{2y}- but not the P_{2x}-purinoceptor. *Br J Pharmacol* 90: 383-391.

Costa M, Furness JB (1983) The origins, pathways and terminations of neurons with VIP-like immunoreactivity in the guinea-pig small intestine. *Neuroscience* 8: 665-676.

Costa M. Furness JB, Humphreys CMS (1986) Apamin distinguishes two types of relaxation mediated by enteric nerves in the guinea-pig gastrointestinal tract. *Naunyn-Schmiedeberg's Arch Pharmacol* 332: 79-88.

Crist JR, He XD, Goyal RK (1992) Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. *J Physiol* 447: 119-131.

D'Amato M, De Beurme FA, Lefebvre RA (1988) Comparison of the effect of vasoactive intestinal polypeptide and non-adrenergic non-cholinergic neurone stimulation in the cat gastric fundus. *Eur J Pharmacol* 152: 71-82.

De Beurme FA, Lefebvre RA (1987) Influence of α-chymotrypsin and trypsin in the non-adrenergic non-cholinergic relaxation in the rat gastric fundus. *Br J Pharmacol* 91: 171-177.

De Beurme FA, Lefebvre RA (1988) Vasoactive intestinal polypeptide as possible mediator of relaxation in the rat gastric fundus. *J Pharm Pharmacol* 40: 711-715.

Den Hertog A., Nelemans A., Van den Akker J. (1989) The inhibitory action of suramin on the P_2-purinoceptor response in smooth muscle cells of the guinea-pig taenia caeci. *Eur J Pharmacol* 166: 531-534.

Ellis JL, Farmer SG (1989a) The effects of vasoactive intestinal peptide (VIP) antagonists, and VIP and peptide histidine isoleucine antisera on non-adrenergic, non-cholinergic relaxations of tracheal smooth muscle. *Br J Pharmacol* 96: 513-520.

Ellis JL, Farmer SG (1989b) Effects of peptidases on non-adrenergic, non-cholinergic inhibitory responses of tracheal smooth muscle: a comparison with effects on VIP- and PHI-induced relaxation. *Br J Pharmacol* 96: 521-526.

Feelish M (1991) The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choise of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J Cardiovasc Pharmacol* 17 (suppl 3): S25-S33.

Goyal RK, Rattan S, Said S (1980) VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory neurones. *Nature* 288: 378-380.

Grider JR, Cable MB, Bitar KN, Said SI, Makhlouf GM (1985) Vasoactive intestinal peptide: relaxant neurotransmitter in the tenia coli of the guinea pig. *Gastroenterology* 89: 36-42.

Gu ZF, Menozzi D, Okamoto A, Maton PN, Bunnett NW (1993) Neutral endopeptidase (EC 3.4.24.11) modulates the contractile effects of neuropeptides on muscle cells from the guinea-pig stomach. *Exper Physiol* 78: 35-48.

Hoyle CHV, Knight GE, Burnstock G (1990) Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br J Pharmacol* 99: 617-621.

Jury J, Jager LP, Daniel EE (1985) Unusual potassium channels mediate nonadrenergic noncholinergic nerve-mediated inhibition in opossum esophagus. *Can J Physiol Pharmacol* 63: 107-112.

Katsoulis S, Clemens A, Schwörer H, Creutzfeldt W, Schmidt WE (1993) PACAP is a stimulator of neurogenic contraction in guinea pig ileum. *Am J Physiol* 265: G295-G302.

Kennedy C (1990) P_1- and P_2-purinoceptor subtypes - an update. *Arch Int Pharmacodyn* 303: 30-50.

Lefebvre RA, Burnstock G (1990). Effect of adenosine triphosphate and related purines in the rat gastric fundus. *Arch Int Pharmacodyn* 303: 199-215.

Maas AJJ, Den Hertog A, Van den Akker J (1980) The action of apamin on the guinea-pig taenia caeci. *Eur J Pharmacol* 67: 265-274.

Nau R, Schafer G, Deacon CF, Cole T, Agoston DV, Conlon JM (1986) Proteolytic inactivation of substance P and neurokinin A in the longitudinal muscle layer of guinea-pig small intestine. *J Neurochem* 47: 856-864.

Pandol SJ, Dharmsathaphorn K, Schoeffield MS, Vale W, Rivier J (1986) Vasoactive intestinal peptide receptor antagonist [4-Cl-D-Phe^6, Leu^{17}]VIP. *Am J Physiol* 250: G553-G577.

Presti ME, Gardner JD (1993) Receptor antagonists for gastrointestinal peptides. *Am J Physiol* 264: G399-G406.

Probert L, De Mey J, Polak JM (1983) Ultrastructural localization of four different neuropeptides within separate populations of p-type nerves in the guinea-pig colon. *Gastroenterology* 85: 1094-1104.

Rhoden KJ, PJ Barnes (1989) Epithelial modulation of NANC and VIP-induced responses: role of neutral endopeptidase. *Eur J Pharmacol* 171: 247-250.

Shuba MF., Vladimirova IA (1980) Effect of apamin on the electrical responses of smooth muscle to adenosine 5'-triphosphate and to non-adrenergic, non-cholinergic nerve stimulation. *Neuroscience* 5: 853-859.

Stark ME, Szurszewski JH (1992) Role of nitric oxide in gastrointestinal and hepatic function and desease. *Gastroenterology* 103: 1928-1949.

Turner JT, Jones SB, Byland DB (1986) A fragment of VIP (10-28), is an antagonist of VIP in the colon carcinoma cell line, HT29. *Peptides* 7: 849-854.

Venugopalan CS, Holmes EP, Malley NA (1990) Evidence for vasoactive intestinal peptide as a mediator of non-adrenergic non-cholinergic neurotransmission in the trachea. *J Auton Pharmacol* 10: 297-304.

Wallenstein S, Zucker CL, Fleiss JL (1980) Some statistical methods useful in circulation research. *Circ Rec* 47: 1-9.

Ward SM, McKeen EM, Sanders KM (1992). Role of nitric oxide in non-adrenergic, non-cholinergic inhibitory junction potentials in canine ileocolonic sphincter. *Br J Pharmacol* 105: 776-782.

Chapter 7

Novel 5-HT_2-like receptor mediates neurogenic relaxation on the guinea-pig proximal colon

M.R. Briejer, L.M.A. Akkermans, R.A. Lefebvre
and J.A.J. Schuurkes

slightly modified: *Eur J Pharmacol*, in press

Abstract 5-hydroxytryptamine (5-HT) contracts the guinea-pig proximal colon *via* 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors, and causes relaxation *via* unknown neurogenic 5-HT receptors. The aim of the current investigation was to characterize these 5-HT receptors.

Ketanserin 0.3 μM and tropisetron 3 μM were continuously present to prevent contractions to 5-HT, and the strips were precontracted with methacholine 0.3 μM. Under these circumstances, 5-HT induced relaxations from 10 nM onwards, yielding a biphasic concentration-response curve. Other tryptamines were also agonists with the following rank order of potency: 5-HT > 5-carboxamidotryptamine (5-CT) = 5-methoxytryptamine (5-MeOT) ≥ α-methyl-5-HT (α-Me-5-HT; partial agonist) > tryptamine (partial agonist). 5-Hydroxytryptophan, 2-methyl-5-HT (2-Me-5-HT) and N-methyl-tryptamine were virtually inactive as agonists. The concentration-response curve to 5-HT was not affected by pargyline (MAO inhibitor), citalopram (5-HT uptake inhibitor), phentolamine (α-adrenoceptor antagonist), a tenfold increase of the ketanserin concentration, or by the 5-HT_4 receptor antagonists 2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino)-ethyl ester (SDZ 205-557) and (1-butyl-4-piperidinylmethyl)-8-amino-7-chloro-1,4-benzodioxan-5-carboxylate (SB 204070). 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT), (5-methoxy-3[1,2,3,6-tetrahydroxypyridin-4-yl]-1H-indole (RU 24969), 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane (WB 4101), 1-(3-chlorophenyl)-piperazine (mCPP), 1-(m-trifluoromethylphenyl)-piperazine (TFMPP), flesinoxan, sumatriptan and 6-chloro-2-(piperazinyl)-pyrazine (MK212) were inactive as 5-HT receptor agonists; some were found to antagonize methacholine-induced contractions. The first phase of the concentration-relaxation curve to 5-HT was inhibited by the following drugs: metergoline (pA_2 = 8.8 ± 0.3, against 5-MeOT 9.3 ± 0.3), methysergide (non-surmountable; NS), methiothepin (NS), spiroxatrine (NS), MK212 (NS), mesulergine (7.8 ± 0.3), mCPP (7.1 ± 0.1), mianserin (7.0 ± 0.4), ritanserin (8.9 ± 0.2), rauwolscine (7.0 ± 0.2), yohimbine (6.2 ± 0.2), 1-(1-naphtyl)-piperazine (1-NAP; 7.7 ± 0.2) and RU 24969 (6.4 ± 0.1), but not by 1-(2-methoxyphenyl)-4-[4-(2-phtalimidobtyl] -piperazine (NAN-190), spiperone, sumatriptan, 8-OH-DPAT and flesinoxan. When compared with pharmacological affinity and efficacy profiles at the currently recognized 5-HT receptor subtypes, the guinea-pig colon high affinity 5-HT receptor correlated only with receptors of the 5-HT_2 group (significantly with 5-HT_{2C} and near-significantly with 5-HT_{2A}). The receptor under study however also displayed pronounced pharmacological differences with these receptors.

It is therefore suggested that the 5-HT receptor under study could be considered a 5-HT_2-like receptor.

Introduction

In the guinea-pig gastrointestinal tract, several receptors for serotonin (5-hydroxytryptamine; 5-HT) have been identified that are involved in motor responses: classical 5-HT_2 receptors mediating smooth muscle contraction (this receptor is currently designated 5-HT_{2A}, Humphrey *et al.*, 1993), and 5-HT_3 and 5-HT_4 receptors on the enteric nerves, which cause an increase in acetylcholine release upon stimulation (Engel *et al.*, 1984; Elswood *et al.*, 1991; Briejer *et al.*, 1993). Furthermore, acetylcholine release-inhibiting prejunctional 5-HT_{1A} receptors have been identified on the myenteric nerves (Fozard & Kilbinger, 1985; Galligan, 1992). However, several studies have appeared, presenting data

on 5-HT receptors in guinea-pig gastrointestinal tissues that cannot be classified according to the 5-HT receptor classification scheme as proposed by Humphrey *et al.* (1993). In the stomach fundus and in the ileum, 5-HT receptors have been identified on the smooth muscle with 5-HT_1-like properties (Feniuk *et al.*, 1983; Kalkman *et al.*, 1986; Kojima *et al.*, 1992). In the proximal colon, neuronal 5-HT receptors were found to mediate relaxation involving nitric oxide (Kojima *et al.*, 1991; Elswood & Bunce, 1992; Briejer *et al.*, 1992; 1995). Classically, responses to 5-HT that are inhibited by the 5-HT_1/5-HT_2 receptor antagonists methiothepin and methysergide, but not by the 5-HT_2 receptor antagonist ketanserin and a 5-HT_3 receptor antagonist like tropisetron, would be designated as mediated by 5-HT_1 receptors (Bradley *et al.*, 1986). Based on these criteria, Kojima (1991) and Elswood & Bunce (1992) suggested that the 5-HT receptor that mediates relaxation in the guinea-pig colon was a 5-HT_1 receptor subtype. As in the mean time many new receptor subtypes were characterized, the 5-HT receptor classification scheme was extended, and new criteria for classification were proposed (Humphrey *et al.*, 1993). According to these novel guidelines, some of the above described "orphan" receptors would probably not be designated 5-HT_1-like.

The aim of the current study was to try and characterize the high affinity 5-HT receptor mediating relaxation of the guinea-pig proximal colon longitudinal muscle. We therefore tested both 5-HT receptor agonists and antagonists and compared observed activity and affinity to literature data, in the view of the novel 5-HT receptor classification scheme.

Materials and methods

Preparation

Dunkin-Hartley guinea pigs of both sexes, weighing 400-600 g, were killed by stunning and decapitation. The proximal colon was removed, and the luminal contents were washed out with De Jalon solution (mM: KCl 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, NaCl 155, D-(+)-glucose 2.8). The mesentery was carefully removed. Starting at the proximal end, about 1 cm distal from the caecum, the colon was divided into four segments of circa 2.5 cm. These intact segments were individually mounted vertically for isotonic measurement into a tissue bath containing 20 ml De Jalon solution. This solution was kept at 37 °C and gassed with 95% O_2, 5 % CO_2. The tissues were subjected to a load of 2 g and allowed to stabilize for half an hour. Ketanserin (0.3 μM) and tropisetron (3 μM) were

continuously present in the De Jalon solution during all experiments to block contractile responses mediated by 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors. After stabilization, the longitudinal muscle was challenged with 0.3 μM methacholine. After washing and 10 min stabilization, the procedure was repeated in order to stabilize the response of the tissue.

Protocols

For the construction of concentration-response curves, drugs were applied directly to the tissue bath (added volume ≤ 1 % of tissue bath volume). Methacholine (0.3 μM) was added to the bath in order to precontract the muscle.

As the relaxations to 5-HT were previously shown to be prone to desensitization (Briejer *et al.*, 1992), non-cumulative concentration-response curves to the agonists were established, beginning 10 min after the administration of methacholine. As soon as a maximum relaxation to a concentration of agonist occurred (after 2 to 4 min), methacholine and the agonist were washed out by replacing the bathing fluid twice and stabilization of 10 min was allowed. This 20 min dosing cycle was repeated applying the next concentration of agonist (ascending order). The response to repetitive administration of methacholine was stable throughout the experiment. Each segment was first challenged with 10 μM 5-HT in the absence of an antagonist (ketanserin and tropisetron present). After washout, this cycle was repeated, applying again 10 μM 5-HT. The relaxation to this latter administration of 5-HT was taken as 100 % relaxation. Consecutively, drugs that might interfere with the agonist under study, were administered, and were left with the preparations for 15 min. Then, non-cumulative concentration-response curves were established, applying the above described 20 min dosing cycle. Drugs were re-added directly after each washout. Of 4 segments that were taken from each animal, one strip was utilized as a control (i.e. one curve per preparation).

For the construction of concentration-response curves to methacholine, a similar protocol was used, but the concentrations were added cumulatively instead of non-cumulatively. Hence, first a curve was established in the absence of the drug to be tested, and then, after washout, drug addition and 15 min stabilization the curve was repeated (2 curves per preparation). Concentration-response curves to methacholine were reproducible.

Data analysis

Means ± standard error of the mean (S.E.) were calculated. Differences between control and treated segments were evaluated with one way analysis of

variance and subsequently Dunnett's *t*-test for multiple comparisons (Wallenstein *et al.*, 1980). The level of significance was set at $P < 0.05$. EC_{50} values were determined with linear regression analysis. Only the first phase of the concentration-response curves to 5-HT (0.01-1 μM) and 5-MeOT (0.1-10 μM) were considered for the calculation of affinity parameters. The maximum effects of the first phases were estimated with a Lineweaver-Burk plot followed by linear regression analysis (Tallarida & Murray, 1981). pA_2 values and their standard errors were estimated with the Schild-Gaddum equation, or were determined with a Schild plot, according to the methods described by Tallarida & Murray (1981). In some cases of non-surmountable antagonism, a pD'_2 value (-log (concentration) of antagonist that causes 50 % depression) was calculated according to Van Rossum (1963).

Chemicals

The following drugs were used: ketanserin tartrate, tropisetron, SB 204070, SDZ 205-557, NAN-190 HCl, spiperone, mCPP, TFMPP cyclohexanesulfamate, 2-methyl-5-hydroxytryptamine, 8-OH-DPAT HBr, phentolamine HCl, sumatriptan, mesulergine HCL, ritanserin, spiroxatrine (Janssen Research Foundation, Belgium), metergoline (Gruppo Montedison, Italy), methiothepin maleate (Hoffman-La Roche, Switzerland), dopamine HCl, 5-hydroxytryptophan, N-methyltryptamine, tryptamine HCl, 5-methoxytryptamine HCl (Janssen Chimica, Belgium), methacholine HCl (E. Merck, Germany), 5-hydroxytryptamine creatinine sulphate (Serva, Germany), paroxetine HCl (Ferrosan, Denmark), citalopram HBr (Lundbeck, Denmark), pargyline HCl (Abbott, USA), 5-carboxamidotryptamine maleate, α-methyl-5-hydroxytryptamine maleate (Cookson Chemicals, UK), 1-(1-naphtyl)piperazine HCl (Lilly, UK), flesinoxan HCl (Duphar, The Netherlands), mianserin HCl (Organon, The Netherlands), methysergide maleate (Sandoz, Switzerland), MK212 HCl (MSD, USA), rauwolscine HCl (Carlrooth, UK), RU 24969 · $^1/_2$ tartrate (Roussel-Uclaf, France), yohimbine HCl (Sigma, Belgium), WB4101 (Amersham, UK). All compounds were dissolved in distilled water, except for the bases, which were dissolved in distilled water acidified with tartaric acid in the stock solution ($pH \geq 3$). The stock solution for 5-HT contained 0.25 μM ascorbic acid. This vehicle had no effect on the strips.

Results

5-HT and analogues

As previously described (Briejer *et al.*, 1992; 1995), 5-hydroxytryptamine induced relaxations from 10 nM onwards, yielding a biphasic concentration-response curve (Figure 7.1). The relaxations reached a maximum effect in 1-2 min, and then decayed in the course of minutes. The first phase ranged from 10 nM to 1 μM (maximum 83 %; pEC_{50} = 7.00 ± 0.04; n = 79), and the second phase ranged from 1 μM to 100 μM. 5-MeOT and 5-CT induced relaxations, yielding a biphasic and a monophasic concentration-response curve, respectively (pEC_{50} values 6.16 ± 0.03 and 6.10 ± 0.02 respectively; n = 22 and 10) (Figure 7.1). α-Me-5-HT and tryptamine also induced relaxations, but with a lower potency and efficacy (Figure 7.1). 5-Hydroxytryptophan, 2-Me-5-HT and N-methyl-tryptamine were virtually inactive (Figure 7.1).

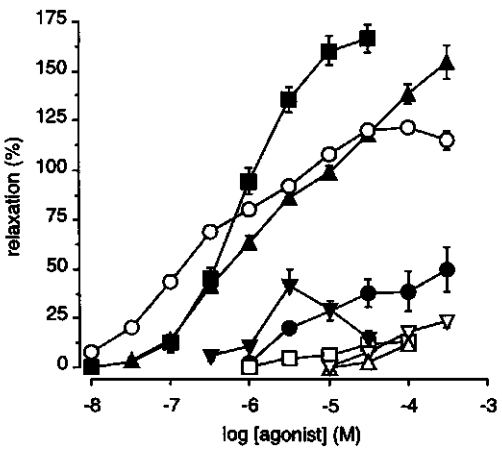


Figure 7.1 Concentration-response curves to 5-hydroxytryptamine (5-HT) (○; n = 79) and its analogues 5-carboxamidotryptamine (■, n = 10), 5-methoxy-tryptamine (▲, n = 22), α-methyl-5-HT (▼, n = 4), tryptamine (●, n = 8), 2-methyl-5-HT (□, n = 4), N-methyltryptamine (△, n = 6) and 5-hydroxytryptophan (▽, n = 4). All experiments were done in the presence of ketanserin 0.3 μM and tropisetron 3 μM, and the strips were precontracted with methacholine 0.3 μM. Relaxations were expressed as a percentage of relaxations induced by 5-HT 10 μM. Points represent the mean ± S.E.

Table 7.1 Intrinsic effects of drugs and effects against 5-HT- and methacholine-induced responses on the guinea-pig colon. All experiments were performed in the presence of ketanserin (0.3 μM) and tropisetron (3 μM).

drug	induces relaxations exceeding	inhibition of contractions[a] to methacholine	inhibition of relaxations to 5-HT	relaxation sensitive to methysergide	L-NNA
8-OH-DPAT	1 μM	pA_2 = 5.2[b]; 40 % depression (100 μM)	1 μM no effect	no	NT
dopamine	100 μM	NT	NT	NT	no
flesinoxan	100 μM	depression 60 % no shift (100 μM)	1 μM no effect	no	NT
mCPP	30 μM	pA_2 ~ 4.5; 30 % depression (100 μM)	NT	no	NT
TFMPP	10 μM	pA_2 ~ 5; 20 % depression (30 μM)	NT	no	NT
MK212	3 μM	pA_2 ~ 5.5	pD_2' = 5.6	no	NT
WB4101	10 μM	NT	NT	no	no
sumatriptan	30 μM	100 μM no effect	1 μM no effect	NT	no[c]
RU 24969	30 μM	pA_2 ~ 4.5; 20 % depression (100 μM)	pA_2 =6.4±0.1	no	no

[a] a pA_2 value was estimated with the Schild equation, if possible; [b] pA_2 value of 8-OH-DPAT 10 μM and 30 μM respectively 5.20 ± 0.01 and 5.20 ± 0.09; [c] relaxations to 5-HT, but not to sumatriptan, were abolished by TTX 0.3 μM; NT = not tested.

Other putative 5-HT receptor agonists

Relaxations to 5-HT are highly sensitive to the NO synthase inhibitor N^G-nitro-L-arginine (L-NNA; 100 μM) (Briejer *et al.*, 1992; 1995) as well as to methysergide (1 μM) (see below). All compounds mentioned in Table 7.1 induced relaxations only at micromolar concentrations, though these relaxations were not sensitive to L-NNA and/or to methysergide. Thus, a mechanism other than 5-HT receptors was suspected to account for the relaxations to these compounds. Therefore, these compounds (except for WB4101) were tested against contractions to methacholine, and it was found that they shifted and/or depressed the concentration-response curve to methacholine (see Table 7.1).

Sumatriptan (100 μM) did not affect the concentration-response curve to methacholine, suggesting even a different mode of action (n = 4; not shown).

Various drugs

Relaxations to 5-HT were not affected by the α-adrenoceptor antagonist phentolamine (1 μM; n = 4) (not shown). The MAO-inhibitor pargyline (1-10 μM) depressed the concentration-response curves to 5-HT, 5-CT and 5-MeOT slightly, but no shift to the left (potentiation) was observed (n = 4; not shown). Incubation with the 5-HT uptake blocker citalopram (1 μM) had no effect (n = 4; not shown) on 5-HT-induced relaxations. Hence, in further experiments no α-adrenoceptor antagonist, MAO-inhibitor or uptake blocker was present.

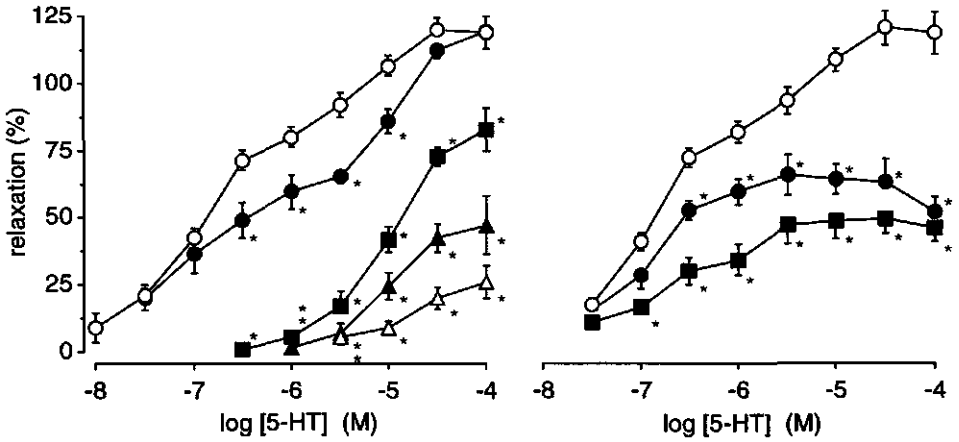


Figure 7.2 Concentration-response curves to 5-HT in the absence (O; n = 6-10) and presence of: (*left panel*) methysergide 0.3 nM (●, n = 4), 3 nM (■, n = 4), 30 nM (▲, n = 6) and 300 nM (Δ, n = 6); (*right panel*) methiothepin 3 nM (●, n = 6) and 30 nM (■, n = 6). All experiments were done in the presence of ketanserin 0.3 μM and tropisetron 3 μM, and the strips were precontracted with methacholine 0.3 μM. Relaxations were expressed as a percentage of relaxations induced by 5-HT 10 μM. Points represent the mean ± S.E. Mean values that are significantly different from control ($P < 0.05$) are marked with an asterisk.

Table 7.2 Estimated pEC_{50} and pA_2 values of 5-HT receptor ligands against the high affinity phase of the concentration-relaxation curve to 5-HT, as compared to literature pEC_{50} values and pA_2 values/binding affinities (pK_i).

agonist	$pEC_{50} \pm$ S.E.	n	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{1D}$	5-HT$_{1E}$	5-HT$_{1F}$	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_{2C}$
5-HT	7.00 ± 0.04	79	7.6	7.8	7.6	7.3	8.1	7.6	8.0-8.4	6.5
5-CT	6.10 ± 0.02	10	8.6	7.9	8.1	4.7	6.0	3.5	6.9-7.6	5.7
5-MeOT	6.16 ± 0.03	22	8.0[a]	6.4[a]	8.4[a]	5.3	5.9[a]	5.5[a]	7.8	7.6[a]
α-Me-5-HT	~ 5.7	4	-	-	-	6.9[a]	6.7[a]	7.3	8.0	7.3
tryptamine	~ 5.5	6	6.8[a]	5.0[a]	7.1[a]	5.6-6.5[a]	5.6[a]	6.0[a]	6.7-7.2	7.3
2-Me-5-HT	i	6	5.8[a]	6.1[a]	5.8[a]	6.1[a]	6.4[a]	< 5[a]	6.2-7.0	6.3[a]

antagonist	$pA_2 \pm$ S.E.	n	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{1D}$	5-HT$_{1E}$	5-HT$_{1F}$	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_{2C}$
ritanserin	8.9 ± 0.2	6	5.2-6.1	4.0-5.8	5.8-6.4	-	-	8.5-9.3	8.3	8.6-9.3
metergoline	8.8 ± 0.3[b]	6	8.1	7.4	9.1	6.0	6.5	8.5[d]	8.5-9.0[d]	10.6[d]
mesulergine	7.8 ± 0.3[g]	6	6.2-6.8	4.9-5.9	5.2-5.4	-	< 5	9.1[d]	7.4	9.1[d]
1-NAP	7.7 ± 0.2	6	7.2	6.6	-	6.7	7.3	7.2	8.9-9.0[d]	8.2
mCPP	7.1 ± 0.1	6	6.5	6.6	5.8	5.5	-	6.7	7.9[d]	7.7
mianserin	7.0 ± 0.4	6	6.0	6.0[d]	6.5[d]	7.0	-	10.1[d]	6.7-7.3	8.0[d]
rauwolscine	7.0 ± 0.2	8	6.9	5.3	7.7	5.4	6.0	6.1	8.5[d]	5.8
RU 24969	6.4 ± 0.1	5	8.1	8.2-8.4	7.3-7.4	7.2	-	5.8-6.0	7.5[d]	6.5-7.3
yohimbine	6.2 ± 0.2	8	6.9	5.5	7.1	5.9-6.4	7.0	6.0	7.9-8.2[d]	4.4
NAN-190	0.3 μM i	6	8.9	6.2	6.7	-	6.7	6.7	-	6.2
sumatriptan	1 μM i	4	6.1-6.6	6.4-6.8	7.2-7.5	5.6-5.7	7.6	3.7	< 5	4.1-5.1
8-OH-DPAT	1 μM i	4	8.6-8.7	4.2-5.8	5.9-6.0	5.5-6.1	5.8	< 5-5.0	5.1-5.4	5.1-5.2
flesinoxan	1 μM i	3	8.8	6.1	6.8	-	-	5.4	-	< 5
spiperone	1 μM i[c]	6	7.2[d]	4.4[d]	4.8[d]	5.0-5.3	< 5	8.8-9.4	< 4-5.5	5.9
ketanserin	3 μM i	4	< 5-5.9	< 5-5.7	5.7-6.0	4.1	< 5	9.3[d]	5.4	6.6[d]
spiroxatrine	nd	6	8.1-8.4	3.6-3.9	5.1	-	-	6.2-6.9	< 5[d]	5.1
MK212	5.6[e]	3	5.3	5.0	-	4.3	-	4.8	< 4-6.4	6.2
methysergide	9.0[e]	4	7.6	5.8	8.4	6.5-7.2	7.5	8.6	7.1-8.2[f]	8.6
methiothepin	7.8[e]	6	7.1	7.3	6.3	6.7-6.9	6.2	8.8	-	7.6

i = inactive, nd = not determined; [a] pK_i binding affinity; [b] pA_2 against 5-MeOT 9.3 ± 0.3 (n = 6), estimated with metergoline 3 nM; [c] depression of second phase of concentration-relaxation curve to 5-HT; [d] pA_2 value instead of pK_i binding affinity; [e] pD'_2 values; [f] $pIC_{50} = 8.7$; [g] pA_2 estimated with 30 nM mesulergine, estimation with 100 nM mesulergine yields a pA_2 of 7.9 ± 0.1 (n = 3). Literature affinity data inferred from: Clineschmidt *et al.*, 1985; Nelson & Taylor, 1986; Cohen & Fludzinski, 1987; Hoyer, 1988; Leonhardt *et al.*, 1989; Van Wijngaarden *et al.*, 1990; Hoyer & Schoeffter, 1991; Kalkman & Fozard, 1991; Foguet *et al.*, 1992; Kursar *et al.*, 1992; Zgombick *et al.*, 1992; Adham *et al.*, 1993; Beer *et al.*, 1993; Gudermann *et al.*, 1993; Wainscott *et al.*, 1993.

5-HT receptor antagonists

Increasing the concentration of ketanserin (a selective 5-HT_{2A} receptor antagonist, Table 7.2) 10-fold to 3 μM, had no significant effect on the concentration-response curve to 5-HT, as compared to the "standard" concentration of 0.3 μM (n = 8; not shown). The 5-HT_4 receptor antagonists SDZ 205-557 (1 μM) or SB 204070 (10 nM) had also no effect (n = 6-7, not shown).

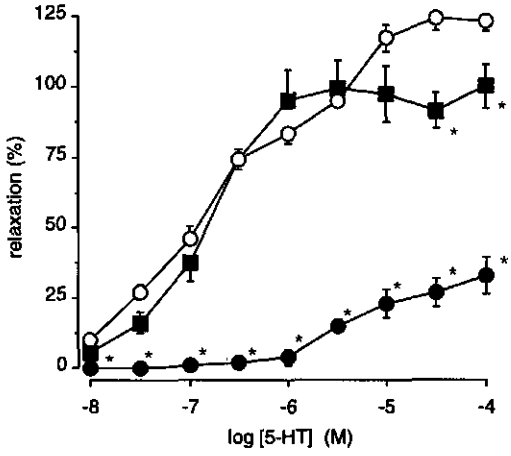


Figure 7.3 Concentration-response curves to 5-HT in the absence (O; n = 6) and presence of: spiroxatrine 0.3 μM (●, n = 6) and spiperone 1 μM (■, n = 6). All experiments were done in the presence of ketanserin 0.3 μM and tropisetron 3 μM, and the strips were precontracted with methacholine 0.3 μM. Relaxations were expressed as a percentage of relaxations induced by 5-HT 10 μM. Points represent the mean ± S.E. Mean values that are significantly different from control ($P < 0.05$) are marked with an asterisk.

The 5-HT_1/5-HT_2 receptor antagonist methysergide (Table 7.2) inhibited both the first and second phase of the concentration-response curve to 5-HT, though to a different extent (Figure 7.2, *left panel*). At 0.3 nM, the first phase was depressed relatively more than the second phase (n = 4), while at 3 nM (n = 4), the first phase was almost blocked. Increasing the concentration methysergide to 30 nM and 300 nM (n = 6), also depressed the second phase to an increasing extent (Figure 7.2, *left panel*). For inhibition of the first phase, a pD'_2 value of 9.0 was estimated. The 5-HT_1/5-HT_2 receptor antagonist methiothepin (Table 7.2) (3 and 30 nM; n = 6) preferentially depressed the second phase of the concentration-response curve to 5-HT, though the first phase was also depressed

(Figure 7.2, *right panel*). The 5-HT$_{1A}$/5-HT$_{2A}$ receptor antagonist spiperone (Table 7.2) (1 μM) only depressed the second phase of the curve to 5-HT (n = 6), leaving the first phase unaffected (Figure 7.3). The 5-HT$_{1A}$ receptor antagonist spiroxatrine (Table 7.2) (0.3 μM) virtually blocked the first phase of the concentration-response curve to 5-HT (n = 6), but at a concentration of 3 nM, it had no effect. Only in the concentration-range coinciding with the second phase, relaxations were not fully blocked by spiroxatrine (0.3 μM) (Figure 7.3).

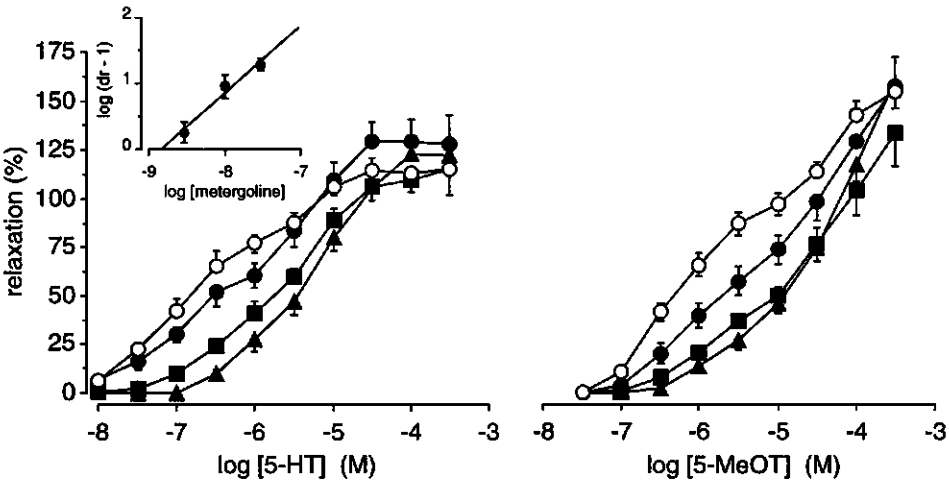


Figure 7.4 Concentration-response curves to 5-HT (*left panel*) and 5-MeOT (*right panel*) in the absence (O; n = 6) and presence of metergoline 3 nM (●, n = 6), 10 nM (■, n = 6) and 30 nM (▲, n = 6). All experiments were done in the presence of ketanserin 0.3 μM and tropisetron 3 μM, and the strips were precontracted with methacholine 0.3 μM. Relaxations were expressed as a percentage of relaxations induced by 5-HT 10 μM. Points represent the mean ± S.E. The inserted figure on the left represents a Schild-plot (dr = dose ratio). The line was fitted with the least squares method, and did not significantly differ from unity. All concentrations caused a significant shift of the EC_{50} of the high affinity phase of the concentration-response curve to 5-HT and 5-MeOT ($P < 0.05$), except for 3 nM against 5-HT ($P < 0.10$).

Neither the selective 5-HT$_{1A}$ receptor antagonists NAN-190 (0.3 μM), flesinoxan (1 μM) and the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (1 μM), nor the moderately selective 5-HT$_{1D}$ receptor agonist sumatriptan (1 μM) (see for selectivities Table 7.2) did affect the relaxations to 5-HT (n = 3-6). Metergoline (5-HT$_1$/5-HT$_2$ receptor antagonist, Table 7.2) (3, 10 and 30 nM, n = 6) inhibited the relaxations to 5-HT and 5-MeOT in a surmountable fashion. As can be seen from Figure 7.4 (*left panel*), metergoline preferentially shifted the first phase of the

concentration-response curves to 5-HT. Schild analysis of the inhibition against 5-HT yielded a slope of 1.01 ± 0.21 (Figure 7.4, *left panel*) and a pA_2 of 8.8 ± 0.3 (constrained at unity: pK_d = 8.9 ± 0.1). Schild analysis of the antagonism against 5-MeOT however (Figure 7.4, *right panel*) yielded a slope (0.58 ± 0.26) that differed from unity. The pA_2 was estimated by using only the shift caused by 3 nM metergoline, and was calculated to be 9.3 ± 0.3.

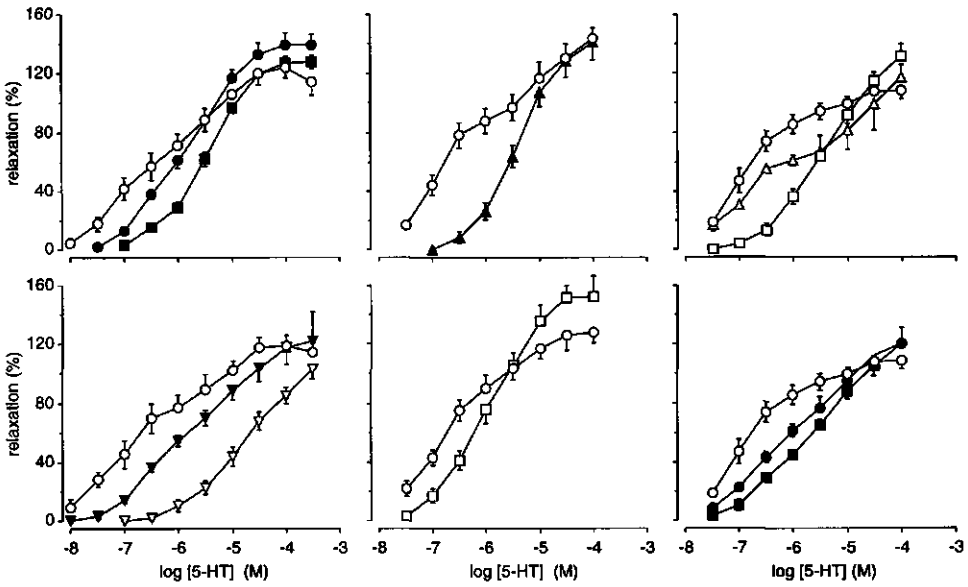


Figure 7.5 Concentration-response curves to 5-HT in the absence (O; n = 5-8) and presence of: *(upper left panel)* yohimbine 1 μM (●, n = 8), rauwolscine 1 μM (■, n = 8); *(upper middle panel)* 1-NAP 0.3 μM (▲, n = 6); *(upper right panel)* MK212 1 μM (Δ, n = 3), mCPP 1 μM (□, n = 6); *(lower left panel)* mianserin 0.3 μM (▼, n = 6), ritanserin 0.1 μM (∇, n = 6); *(lower middle panel)* RU 24969 1 μM (□, n = 5); *(lower right panel)* mesulergine 0.03 μM (●, n = 6) and 0.1 μM (■, n = 3). All experiments were done in the presence of ketanserin 0.3 μM and tropisetron 3 μM, and the strips were precontracted with methacholine 0.3 μM. Relaxations were expressed as a percentage of relaxations induced by 5-HT 10 μM. Points represent the mean ± S.E. All antagonists caused a significant shift of the EC_{50} of the high affinity phase of the concentration-response curve to 5-HT ($P < 0.05$).

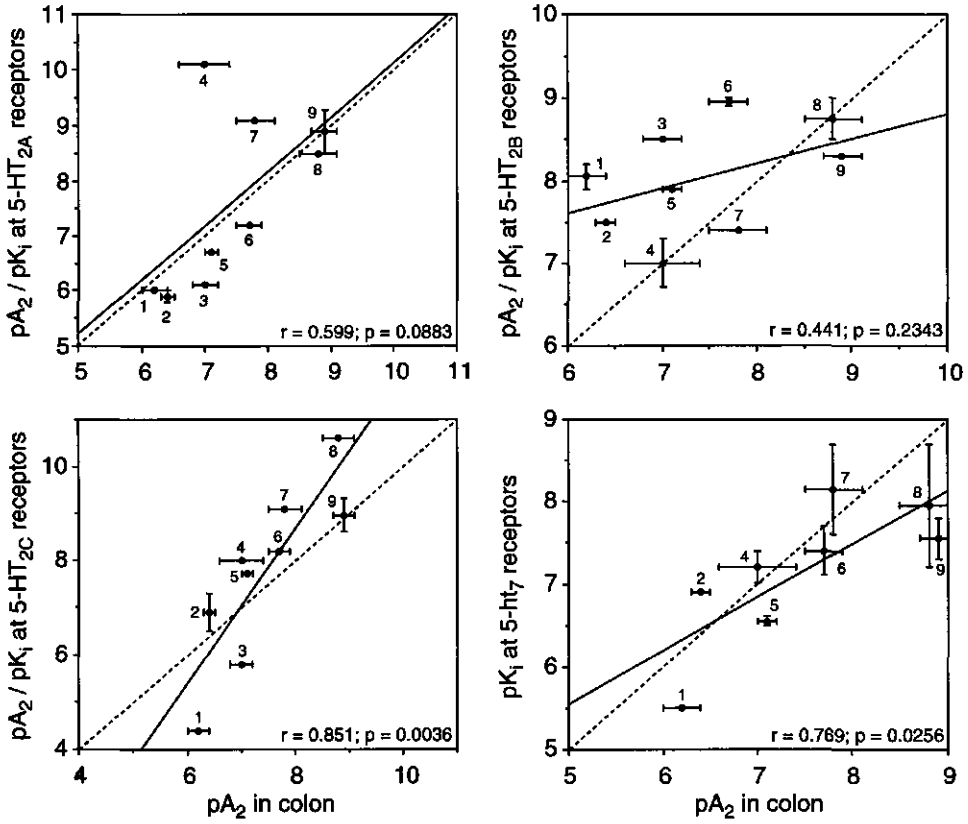


Figure 7.6 Correlations of affinity values of antagonists for 5-HT$_{2A}$,5-HT$_{2B}$, 5-HT$_{2C}$ and 5-ht$_7$ receptors on one hand, and for the relaxation-mediating 5-HT receptor in the guinea-pig colon on the other hand. pA_2 values (± S.E.) in the colon of 8-9 compounds (X-axis) are plotted against pA_2 values or, if not available, pK_i binding values inferred from the literature (see also Table 7.2) on the Y-axis. In case more than 2 values were found in literature, the value in between the extremes was used (the error bar represents the extreme values found in literature). The lines were fitted with the least squares method (*r* represents the correlation coefficient) and analysed statistically with ANOVA followed by the *F*-test for significance of correlation, denoted with *p*. Data were obtained from the following compounds: yohimbine (1), RU 24969 (2), rauwolscine (3), mianserin (4), mCPP (5), 1-NAP (6), mesulergine (7), metergoline (8) and ritanserin (9). The *upper left panel* shows the correlation with 5-HT$_{2A}$ receptor affinity values, the *upper right panel* with 5-HT$_{2B}$ receptors, *lower left panel* with 5-HT$_{2C}$ receptors and the *lower right panel* with 5-ht$_7$ receptors.

Yohimbine (1 μM), rauwolscine (1 μM), 1-NAP (0.3 μM), mianserin (0.3 μM), ritanserin (0.1 μM), RU 24969 (1 μM), mesulergine (0.03 and 0.1 μM) and mCPP (1 μM) (compounds that all interfere with 5-HT$_1$ and/or 5-HT$_2$ receptors with variable selectivities: see Table 7.2 for affinities) (n = 5-8) inhibited the relaxations

to 5-HT surmountably, causing a shift of the first phase of the concentration-response curve to 5-HT (Figure 7.5; for estimated pA_2 values see Table 7.2). MK212 (1 μM) caused a depression (rather than a shift to the right) of the first but not the second phase of the concentration-response curve to 5-HT (n = 3; Figure 7.5, *upper right panel*).

Of the antagonists tested, 9 allowed estimation of a pA_2 value. These values correlated near significantly with literature affinity values for 5-HT_{2A} receptors (r = 0.599; P = 0.0883; slope = 0.983; n = 9) and significantly for 5-HT_{2C} receptors (r = 0.851; P = 0.0036; slope = 1.648; n = 9), as shown in Figure 7.6 (*left panels*). No significant correlation was found with affinity values for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2B} receptors (see Figure 7.6, *upper right panel*). Comparison of the affinities of antagonists in the colon with those for the recently cloned 5-ht_{5A}, 5-ht_{5B}, 5-ht_6 and 5-ht_7 receptors yielded only a significant correlation with affinity values for 5-ht_7 receptors (r = 0.769; P = 0.0256; slope = 0.647; n = 8) (Figure 7.6, *lower right panel*).

Discussion

The concentration-response curve to 5-HT in the guinea-pig colon preparation was biphasic, suggesting that two receptors are involved. Though the author did not make special reference, the concentration-relaxation curve to 5-HT in the similar preparation as used by Kojima (1991) also was biphasic, with an inflection at a similar concentration of 5-HT. As compared to our experimental circumstances (intact segments, 5-HT_2, 5-HT_3 and 5-HT_4 receptors blocked), Kojima used strips of which the mucosa was removed, atropine was present in the bath solution to prevent cholinergic contractions *via* 5-HT_3 and 5-HT_4 receptors and ketanserin (10 μM) to block 5-HT_2 receptors, and the strips were not precontracted (Kojima, 1991). The observed differential inhibition of these two phases, notably by methysergide, methiothepin, spiroxatrine, spiperone, but also by other compounds, also suggests a two receptor system. As the low affinity receptor, which mediates the second phase, is much more difficult to quantatively analyze, the receptor characterization as described below focussed only on the 5-HT receptor that accounts for the first, high affinity phase of the concentration-response curve to 5-HT.

In the guinea-pig gut, 5-HT_{1A} receptors are known to be present on the enteric neurons (Fozard & Kilbinger, 1985; Galligan, 1992). For 5-HT_{1A} receptors, some selective compounds are available (see Table 7.2), e.g. 8-OH-DPAT and

flesinoxan, which are agonists (Fozard & Kilbinger, 1985; Schipper *et al.*, 1990), and NAN-190, spiperone and spiroxatrine, which are antagonists (though in some systems they behave as partial agonists) (Nelson & Taylor, 1986; Pauwels *et al.*, 1993). At relevant concentrations (see Table 7.2), however, these compounds showed no consistent effects, and spiperone could only inhibit the second phase of the concentration-response curve to 5-HT. These results suggest that 5-HT_{1A} receptors do not play a role in the 5-HT-induced relaxations. 5-HT_{1B} and 5-HT_{1D} receptors have not (yet) been shown to be present in the gastrointestinal tract. The current results do also not provide evidence for their presence. Compounds that are known to interact with 5-HT_{1B} and/or 5-HT_{1D} receptor subtypes, such as RU 24969, yohimbine, rauwolscine, sumatriptan, metergoline, methysergide and methiothepin (Schoeffter & Hoyer, 1989; Hoyer & Schoeffter, 1991; Miller *et al.*, 1992), had no effects that were consistent with the affinities reported in literature (see Table 7.2). Functional correlates of the more recently discovered 5-HT_{1E} and 5-HT_{1F} receptors have not been encountered yet, and no selective antagonists at 5-HT_{1E} and 5-HT_{1F} receptors (Leonhardt *et al.*, 1989; Zgombick *et al.*, 1992; Adham *et al.*, 1993; Gudermann *et al.*, 1993) are available. The affinities as available from the literature are too scarce to provide decisive evidence as to whether either 5-HT_1 receptor subtype is involved. However, 5-CT is more than 100 times less potent than 5-HT at 5-HT_{1E} or 5-HT_{1F} receptors, which was not the case in our model, rendering involvement of these 5-HT_1 receptor subtypes rather unlikely (see also Table 7.2). At classical 5-HT_1 receptors, 5-CT is more potent than 5-HT, which was one of the criteria for designating a 5-HT receptor 5-HT_1 (Bradley *et al.*, 1986; Table 7.2). In the colon, however, 5-CT was less potent than 5-HT. Hence, taken together, the above described results do not provide evidence that the relaxations to 5-HT in the guinea-pig colon are mediated by a known 5-HT_1 receptor subtype.

Ritanserin and metergoline were potent antagonists of the 5-HT-induced relaxations, and mesulergine, yohimbine, rauwolscine, mCPP and mianserin were moderately potent antagonists. Comparing these data with literature receptor affinity values suggests that a 5-HT_2 receptor subtype could be involved (see Table 7.2). The affinities of 9 antagonists that were tested showed a near significant correlation with literature affinities for 5-HT_{2A} receptors, suggesting some resemblance with this receptor subtype. The potent 5-HT_{2A} receptor antagonists ketanserin and spiperone did however not affect the concentration-response curve to 5-HT. Hence the receptor under study is not identical to the 5-HT_{2A} receptor subtype. The 5-HT_{2B} and 5-HT_{2C} receptors are rather insensitive to inhibition by spiperone and ketanserin (Cohen & Wittenauer, 1985; Hoyer &

Schoeffter, 1991; Foguet *et al.*, 1992; Growcott *et al.*, 1993; Wainscott *et al.*, 1993) as are the relaxations described here in the guinea-pig colon (see Table 7.2; Kojima, 1991; Elswood & Bunce, 1992). In the rat fundus, the 5-HT_{2B} receptor is located on the smooth muscle and mediates contraction. In the guinea-pig colon, on the other hand, 5-HT acts on the nerves, as the relaxations are readily blocked by the neurotoxin tetrodotoxin (Briejer *et al.*, 1992). This is not an argument to exclude that the receptors are similar, as, for example, smooth muscle 5-HT_4 receptors in the rat gastrointestinal tract cause relaxation, while in the guinea-pig they are neurogenic and mediate contraction (Elswood *et al.*, 1991; Reeves *et al.*, 1991; Baxter *et al.*, 1991; Briejer *et al.*, 1993). Though there are some antagonists that have similar affinities both for the colon receptor and the 5-HT_{2B} receptor (see Table 7.2: ritanserin, metergoline, mesulergine), there are however also great differences (see Table 7.2: 1-NAP, rauwolscine, yohimbine). Spiroxatrine has been reported to have no effect on rat fundus contractions to 5-HT up to a concentration of respectively 1 μM (Cohen & Fludzinski, 1987), while in our preparation it strongly inhibited the 5-HT-induced relaxations at 0.3 μM. These authors also found that mianserin up to 0.3 μM did not affect contractions in the rat fundus to 5-HT (Cohen & Fludzinski, 1987), though it was active in our preparation, with a pA_2 value estimated to be 7.0, which is in agreement with receptor binding data (see Table 7.2). α-Me-5-HT was a poor agonist in the colon, while in the rat fundus it is a full and potent agonist (Kalkman & Fozard, 1991; Growcott *et al.*, 1993). Methysergide seemed to have a higher affinity for the colon receptor than for the 5-HT_{2B} receptor (Table 7.2), but in the colon it behaved as a non-competitive antagonist, which makes it difficult to calculate a reliable affinity parameter. Comparison of antagonist affinities at the rat fundus receptor (5-HT_{2B}) and the guinea-pig colon receptor showed no significant correlation. There was however a significant correlation with 5-HT_{2C} receptor affinities, but also here some differences with the colon receptor were apparent. The rank order of potency at 5-HT_{2C} receptors is α-Me-5-HT > 5-HT > 5-CT (Hoyer & Schoeffter, 1991), while in the colon the potency order was 5-HT > 5-CT = α-Me-5-HT, demonstrating especially the difference with respect to α-Me-5-HT. Yohimbine and rauwolscine have only low affinity for 5-HT_{2C} receptors, but in our model however, yohimbine and rauwolscine showed moderate affinity. The affinities of metergoline and mesulergine do also not quite correspond for the colon receptor and for 5-HT_{2C} receptors. These differences are reflected in the steep slope of the fitted line in Figure 7.6. Hence, the 5-HT receptor under study resembles most the 5-HT_{2C} receptor, but its characteristics are not identical.

RU 24969, 8-OH-DPAT, sumatriptan and MK212 (and others: Table 7.1) induced methysergide- and/or L-NNA-insensitive relaxations in our preparation, while those to 5-HT are blocked by methysergide or L-NNA (this paper; Briejer *et al.*, 1992). Relaxations to RU 24969, 8-OH-DPAT and MK212 were shown to be caused by antagonism of the methacholine-induced precontraction. Galligan (1992) also showed that in the guinea-pig ileum, 8-OH-DPAT blocks histamine and muscarinic (pA_2 = 5.5; in our preparation pA_2 = 5.2) receptors. Though the compounds might also induce part of the relaxation *via* the 5-HT receptor under study, the non-specific effects would mask them. Affinity estimates could however be assessed by using them as an antagonist at a concentration that would induce no non-specific effects (see Table 7.1 and 7.2).

Recently, a number of novel 5-HT receptors have been cloned, provisionally designated 5-ht$_{5A}$ (REC17, 5-ht$_{5\alpha}$), 5-ht$_{5B}$ (MR22, 5-ht$_{5\beta}$), 5-ht$_6$ and 5-ht$_7$ (PCT65, REC20, 5-ht$_X$) (Plassat *et al.*, 1992, 1993; Matthes *et al.*, 1993; Erlander *et al.*, 1993; Monsma *et al.*, 1993; Ruat *et al.*, 1993a, b; Shen *et al.*, 1993; Lovenberg *et al.*, 1993; Bard *et al.*, 1993). Comparison of receptor binding affinities and pEC_{50} values in cells in which such receptors were expressed showed a significant correlation with the 5-ht$_7$ receptor. Northern blot analysis revealed the presence of 5-ht$_7$ receptor gene transcripts in mouse and human, but not rat, gut (Ruat *et al.*, 1993a, b; Plassat *et al.*, 1993; Shen *et al.*, 1993; Lovenberg *et al.*, 1993; Bard *et al.*, 1993). No functional correlates have yet been found of the putative 5-ht$_7$ receptor and relevant pharmacological data is too scarce to allow any thorough comparison of the putative 5-ht$_7$ receptor and the receptor currently under study.

In the guinea-pig ileum, a smooth muscle receptor mediates relaxations to 5-HT. These responses could be inhibited by metergoline, spiperone and methysergide, but with quite different pA_2 values as compared to the current results (Feniuk *et al.*, 1983, 1984; Kalkman *et al.*, 1986), which suggests that the receptor involved is of another subtype. Also in the guinea-pig stomach fundus circular muscle, 5-HT induces relaxations *via* a smooth muscle receptor (Kojima *et al.*, 1992). Here, the rank order of potency was 5-CT >> 5-HT = 5-MeOT > 5-Me-5-HT; TFMPP and 8-OH-DPAT were partial agonists, and α-Me-5-HT, 2-Me-5-HT and sumatriptan were virtually inactive. The relaxations to 5-HT in this preparation were inhibited by methiothepin and mianserin, but also by ketanserin and spiperone. These data indicate that the 5-HT receptor in the guinea-pig stomach fundus is also different from the 5-HT receptor in the colon.

It is concluded that the neuronal 5-HT receptor that mediates the first phase of the concentration-response curve to 5-HT (NO-mediated part) is not a known 5-HT receptor, and could be considered a 5-HT_2 -like receptor.

References

Adham N, Kao HT, Schlechter LE,. Bard J, Olsen M, Urquhart D, Durkin M, Hartig PR, Weinshank RL, Branchek TA (1993) Cloning of another human serotonin receptor (5-HT_{1F}): a fifth 5-HT_1 receptor subtype coupled to the inhibition of adenylate cyclase. *Proc Natl Acad Sci USA* 90: 408-412.

Bard JA, Zgombick J, Adham N, Vaysse P, Branchek TA, Weinshank RL (1993) Cloning of a novel human serotonin receptor (5-HT_7) positively linked to adenylate cyclase. *J Biol Chem* 268: 23422-23426.

Beer MS, Middlemiss DN, McAllister G, (1993) 5-HT_1-like receptors: six down and still counting. *Trends Pharmacol Sci* 14: 228-231.

Bradley PB, Engel G, Feniuk W, Fozard JR, Humphrey PPA, Middlemiss DN, Mylecharane EJ, Richardson BP, Saxena PR (1986) Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology* 25: 563-576.

Baxter GS, Craig DA, Clarke DE (1991) 5-Hydroxytryptamine$_4$ receptors mediate relaxation of the rat oesophageal tunica muscularis mucosae. *Naunyn-Schmiedeberg's Arch Pharmacol* 343: 439-446.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1992) Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*. *Br J Pharmacol* 107: 756-761.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1993) Pharmacological characterization of 5-hydroxytryptamine receptor types in the guinea-pig proximal colon. *J Gastroint Motil* 5: 239-246.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1995) 5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide. *Naunyn-Schmiedeberg's Arch Pharmacol* 351: in press.

Clineschmidt BV, Reiss DR, Pettibone DJ, Robinson JL (1985) Characterization of 5-hydroxytryptamine receptors in rat stomach fundus. *J Pharmacol Exp Ther* 235: 696-708.

Cohen ML, Wittenauer LA (1985) Relationship between serotonin and tryptamine receptors in the rat stomach fundus. *J Pharmacol Exp Ther* 233: 75-79.

Cohen ML, Fludzinski LA (1987) Contractile serotonergic receptor in rat stomach fundus. *J Pharmacol Exp Ther* 243: 264-269.

Elswood CJ, Bunce KT (1992) Investigation of the 5-HT receptor mediating relaxation in guinea-pig proximal colon. *J Pharm Pharmacol* 44: 264-266.

Elswood CJ, Bunce KT, Humphrey PPA (1991) Identification of putative 5-HT_4 receptors in guinea-pig ascending colon. *Eur J Pharmacol* 196: 149-155.

Engel G, Hoyer D, Kalkman HO, Wick MB (1984) Identification of 5-HT_2 receptors on longitudinal muscle of the guinea pig ileum. *J Receptor Res* 4: 113-126.

Erlander MG, Lovenberg TW, Baron BM, de Lecea L, Danielson PE, Racke M, Slone L, Siegel BW, Foye FE, Cannon K, Burns JE, Sutcliffe JG (1993) Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. *Proc Natl Acad Sci USA* 90: 3452-3456.

Feniuk W, Humphrey PPA, Watts AD (1983) 5-Hydroxytryptamine-induced relaxation of isolated mammalian smooth muscle. *Eur J Pharmacol* 96: 71-78.

Feniuk W, Humphrey PPA, Watts AD (1984) 5-Carboxamidotryptamine - a potent agonist at 5-hydroxytryptamine receptors mediating relaxation, *Br J Pharmacol* 82: 209P.

Foguet M, Hoyer D, Pardo LA, Parekh A, Kluxen FW, Kalkman HO, Stühmer W, Lübbert H (1992) Cloning and functional characterization of the rat stomach fundus serotonin receptor. *EMBO J* 11: 3481-3487.

Fozard JR Kilbinger HH (1985) 8-OH-DPAT inhibits transmitter release from guinea-pig enteric cholinegic neurones by activating 5-HT$_{1A}$ receptors. *Br J Pharmacol* 86: 601P

Galligan JJ (1992) Differential inhibition of cholinergic and noncholinergic neurogenic contractions by 5-hydroxytryptamine$_{1A}$ receptor agonists in guinea pig ileum. *J Pharmacol Exp Ther* 260: 306-312.

Growcott JW, Cox B, Blackburn TP (1993) A pharmacological comparison of the receptors mediating contractile responses to 5-hydroxytryptamine in the rat isolated caudal artery and fundic strip. *J Pharm Pharmacol* 45: 876-881.

Gudermann T, Levy FO, Birnbaumer M, Birnbaumer L, Kaumann AJ (1993) Human S31 serotonin receptor clone encodes a 5-hydroxytryptamine$_{1E}$-like serotonin receptor. *Mol Pharmacol* 43: 412-418.

Hoyer D (1988) Functional correlates of serotonin 5-HT$_1$ recognition sites. *J Receptor Res* 8: 59-81.

Hoyer D, Schoeffter P (1991) 5-HT receptors: subtypes and second messengers. *J Receptor Res* 11: 197-214.

Humphrey PPA, Hartig P, Hoyer D (1993) A proposed new nomenclature for 5-HT receptors. *Trends Pharmacol Sci* 14: 233-236.

Kalkman HO, Engel G, Hoyer D (1986) Inhibition of 5-carboxamidotryptamine-induced relaxation of guinea-pig ileum correlates with [^{125}I]-LSD binding. *Eur J Pharmacol* 129: 139-145.

Kalkman HO, Fozard JR (1991) Further definition of the 5-HT receptor mediating contraction of rat stomach fundus: relation to 5-HT$_{1D}$ recognition sites. In: *Serotonin: Molecular biology, receptors and functional effects* (Fozard JR, Saxena PR Eds.), Birkhäuser Verlag Basel/Switzerland, pp. 153-160.

Kojima S (1991) Characterization of 5-hydroxytryptamine-induced relaxations of guinea-pig proximal colon. *Arch Int Pharmacodyn* 313: 23-32.

Kojima S, Ishizaki R, Shimo Y (1992) Investigation into the 5-hydroxytryptamine-induced relaxation of the circular smooth muscle of guinea-pig stomach fundus. *Eur J Pharmacol* 224: 45-49.

Kursar JD, Nelson DL, Wainscott DB, Cohen ML, Baez M (1992) Molecular cloning, functional expression, and pharmacological characterization of a novel receptor (5-hydroxytryptamine$_{2F}$) from rat stomach fundus. *Mol Pharmacol* 42: 549-557.

Leonhardt S, Herrick-Davis K, Titeler M (1989) Detection of a novel serotonin receptor subtype (5-HT$_{1E}$) in human brain: interaction with a GTP-binding protein. *J Neurochem* 53: 465-471.

Lovenberg TW, Baron BM, de Lecea L, Miller JD, Prosser RA, Rea MA, Foye PE, Racke M, Slone AL, Siegel BW, Danielson PE, Sutcliffe JG, Erlander MG (1993) A novel adenylyl cyclsae-activating serotonin receptor (5-HT$_7$) implicated in the regulation of mammalian circadian rhythms, *Neuron* 11: 449-458.

Matthes H, Boschert U, Amlaiky F, Plassat JL, Muscatelli F, Mattei MG, Hen R (1993) Mouse 5-hydroxytryptamine$_{5A}$ and 5-hydroxytryptamine$_{5B}$ receptors define a new family of serotonin receptors: cloning, functional expression, and chromosomal localization. *Mol Pharmacol* 43: 313-319.

Miller KJ, King A, Demchyshyn L, Niznik H, Teitler M (1992) Agonist activity of sumatriptan and metergoline at the human 5-$HT_{1D\beta}$ receptor: further evidence for a role of the 5-HT_{1D} receptor in the action of sumatriptan. *Eur J Pharmacol (Mol Pharmacol Sect)* 227: 99-102.

Monsma FJ, Shen Y, Ward RP, Hamblin MW, Sibley DR (1993) Cloning and functional expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Mol Pharmacol* 43: 320-327.

Nelson DL, Taylor EW (1986) Spiroxatrine: a selective serotonin$_{1A}$ receptor antagonist. *Eur J Pharmacol* 124: 207-208.

Pauwels PJ, Van Gompel P, Leysen JE (1993) Activity of serotonin (5-HT) receptor agonists, partial agonists and antagonists at cloned human 5-HT_{1A} receptors that are negatively coupled to adenylate cyclase in permanently transfected HELA cells. *Biochem Pharmacol* 45: 375-383.

Plassat JL, Boschert U, Amkaily N, Hen R (1992) The mouse 5-HT_5 receptor reveals a remarkable heterogeneity within the 5-HT_{1D} receptor family. *EMBO J* 11: 4779-4786.

Plassat JL, Amlaiky N, Hen R (1993) Molecular cloning of a mammalian receptor that activates adenylate cyclase. *Mol Pharmacol* 44: 229-236.

Reeves JJ, Bunce KT, Humphrey PPA (1991) Investigation into the 5-hydroxytryptamine receptor mediating smooth muscle relaxation in the rat oesophagus. *Br J Pharmacol* 102: 1067-1072.

Ruat M, Traiffort E, Leurs R, Tarvidel-Lacombe J, Diaz J, Arrang JM, Schwartz JC (1993a) Molecular cloning, characterization, and localization of a high affinity serotonin receptor (5-HT_7) activating cAMP formation. *Proc Natl Acad Sci USA* 90: 8547-8551.

Ruat M, Traiffort E, Arrang JM, Tarvidel-Lacombe J, Diaz J, Leurs R, Schwartz JC (1993b) A novel rat serotonin (5-HT_6) receptor: molecular cloning, localization and stimulation of cAMP accumulation. *Biochem Biophys Res Commun* 193: 268-276.

Schipper J, Tulp M, Sijbesma H, Krijzer F, Mos J, v.d. Heyden J, Olivier B (1990) Preclinical evaluation of flesinoxan as a potential anxiolytic and antidepressant drug. *Eur J Pharmacol* 183: 2059-2060.

Schoeffter P, Hoyer D (1989) 5-Hydroxytryptamine 5-HT_{1B} and 5-HT_{1D} receptors mediating inhibition of adenylate cyclase activity. Pharmacological comparison with special reference to the effects of yohimbine, rauwolscine and some β-adrenoceptor antagonists. *Naunyn-Schmiedeberg's Arch Pharmacol* 340: 285-292.

Shen Y, Monsma FJ, Metcalf MA, Jose PA, Hamblin MW, Sibley DR (1993) Molecular cloning and expression of a 5-hydroxytryptamine$_7$ serotonin receptor subtype. *J Biol Chem* 268: 18200-18204.

Tallarida RJ, Murray RB (1981) *Manual of pharmacologic calculations with computer programs.* Springer-Verlag, New York-Heidelberg-Berlin.

Van Rossum JJ (1963) Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch Int Pharmacodyn Ther* 143: 299-330.

Van Wijngaarden I, Tulp MTM, Soudijn W (1990) The concept of selectivity in 5-HT receptor research. *Eur J Pharmacol (Mol Pharmacol Sect)* 188: 301-312.

Wainscott DB, Cohen ML, Schenk KW, Audia JE, Nissen JS, Baez M, Kursar JD, Lucaites VL, Nelson DL (1993) Pharmacological characteristics of the newly cloned rat 5-hydroxytryptamine$_{2F}$ receptor. *Mol Pharmacol* 43: 419-426

Wallenstein S, Zucker CL, Fleiss JL (1980) Some statistical methods useful in circulation research. *Circ Res* 47: 1-9.

Zgombick JM, Schechter LE, Macchi M, Hartig PR, Branchek TA, Weinshank RL (1992) Human gene S31 encodes the pharmacologically defined serotonin 5-hydroxytryptamine$_{1E}$ receptor. *Mol Pharmacol* 42: 180-185.

Chapter 8

Cisapride and structural analogs selectively enhance 5-hydroxytryptamine (5-HT)-induced purinergic neurotransmission in the guinea pig proximal colon

M.R. Briejer, G.J. Veen, L.M.A. Akkermans,
R.A. Lefebvre and J.A.J. Schuurkes

slightly modified: *J Pharmacol Exp Ther*, in press

Abstract. In the guinea pig proximal colon, 5-HT stimulates neuronal 5-HT_1-like receptors to induce relaxations that are mediated by nitric oxide (NO) and adenosine 5'-triphosphate (ATP). In the current study, the effects of cisapride and structural analogs on these 5-HT-induced relaxations were investigated.

In the continuous presence of ketanserin (0.3 μM) and tropisetron (3 μM) to block contractions *via* 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors, 5-HT induced relaxations yielding a biphasic concentration-response curve. Cisapride (0.1-1 μM) enhanced the second phase of the concentration-response curve to 5-HT by about 20-40 %, while, from 0.3 μM onwards it inhibited the first phase. Also in the presence of cisapride (0.3 μM), tetrodotoxin (TTX; 0.3 μM) abolished the relaxations to 5-HT. Cisapride (0.3 μM) did not affect the concentration-response curves to isoprenaline, nitroglycerin, nitroprusside or exogenous ATP, demonstrating its specificity. The 5-HT-relaxation enhancing effects of cisapride were not mimicked by phentolamine (1 μM), NAN-190 (0.03 μM), spiperone (1 μM), citalopram (0.3 μM), paroxetine (0.3 μM), pargyline (100 μM) or SDZ 205-557 (0.3 μM). In the presence of the inhibitor of NO synthesis, N^G-nitro-L-arginine (L-NNA; 100 μM), cisapride (0.3 μM) still enhanced the remaining relaxations to 5-HT (2-3 fold). However, in the presence of the P_2-purinoceptor antagonist suramin (300 μM), cisapride could not enhance the relaxations to 5-HT. In the presence of L-NNA, the cisapride-enhanced relaxations to 5-HT could be inhibited by about 90 % by suramin.

Conclusion: In the guinea pig colon, cisapride selectively facilitates the suramin-sensitive, ATP-mediated, part of the relaxation to 5-HT *via* an unidentified effect on intramural nerves.

Introduction

The 2-methoxy-4-amino-5-chloro-substituted benzamides are used in the treatment of impaired gastrointestinal motility, which manifests in disorders like gastro-esophageal reflux, gastroparesis, functional dyspepsia and constipation (Reyntjens *et al.*, 1986; Müller-Lissner, 1987; Reynolds & Putnam, 1992). These prokinetics are represented by cisapride, metoclopramide, clebopride and cinitapride (which is a 2-ethoxy-4-amino-5-nitro-substituted benzamide) (Pinder *et al.*, 1976; Massingham *et al.*, 1985; Reynolds & Putnam, 1992). Renzapride, dazopride and zacopride are not used in the clinic, but have similar gastrointestinal motility stimulating properties (Reynolds & Putnam, 1992; Gullikson *et al.*, 1992). The mechanism of prokinetic action is however still not completely understood. A common feature of the prokinetic benzamides is their agonist action at 5-hydroxytryptamine$_4$ (5-HT_4) receptors (see: Bockaert *et al.*, 1992; Ford & Clarke, 1993), which is thought to cause facilitation of cholinergic (and possibly also non-cholinergic) excitatory neurotransmission (Taniyama *et al.*, 1991; Kilbinger & Wolf, 1992; Bingham & Andrews, 1992). This has been put forward as the mechanism by which motility is enhanced (see: Tonini *et al.*, 1991). However, in a number of tissues, this mechanism does not seem to apply. For example, in the canine gastric antrum, cisapride enhanced field stimulation-

induced cholinergic contractions *via* a mechanism that was not sensitive to 5-HT_4 receptor blockade (De Ridder & Schuurkes, 1993), and in human colon, cisapride did even not enhance the release of acetylcholine (Burleigh & Trout, 1985). In electrophysiological experiments with guinea pig AH/type 2 myenteric neurons, cisapride has been shown to enhance EPSPs *via* 5-HT_4 receptors (see Tonini *et al.*, 1991), but direct 5-HT_4 receptor-mediated responses could not be demonstrated (Nemeth *et al.*, 1985; Mawe *et al.*, 1989; Wade *et al.*, 1991). On such neurons, 5-HT stimulates, next to 5-HT_{1A} and 5-HT_3 receptors, putative 5-HT_{1P} receptors which mediate the slow prolonged depolarization. Some of the above prokinetic benzamides interfere with this 5-HT_{1P} receptor-mediated response, either as agonist (S-zacopride) or antagonist (cisapride, R-zacopride, renzapride) (Nemeth *et al.*, 1985; Mawe *et al.*, 1989; Wade *et al.*, 1991). Enhancement of gastric emptying in mice has been associated with antagonism of 5-HT_{1P} receptors on inhibitory neurons (Mawe *et al.*, 1989).

Previously, it has been shown that 5-HT induces relaxations of the guinea pig colon through the stimulation of a neuronal 5-HT_1-like receptor (Kojima, 1991; Elswood & Bunce, 1992; Briejer *et al.*, 1992). These relaxations were highly sensitive to inhibition of nitric oxide synthesis (Briejer *et al.*, 1992) and were moderately inhibited by suramin and apamin, which were shown to selectively inhibit relaxations to ATP (Briejer *et al.*, 1995). Here we report on the facilitating effects of cisapride and some other prokinetic benzamides on the suramin-sensitive, but not the NO-mediated, part of the relaxation to 5-HT on the guinea pig colon. The mechanism by which this is accomplished was investigated.

Materials and methods

Tissue preparation

Dunkin-Hartley guinea pigs of both sexes, weighing 400-600 g, were killed by stunning and decapitation. The proximal colon was removed, and the luminal contents were washed out with De Jalon solution (mM: KCl 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, NaCl 155, glucose 2.8). The mesentery was carefully removed. Starting at the proximal end, about 1 cm distal from the caecum, the colon was divided into four segments of circa 2.5 cm. These intact segments were individually mounted vertically for isotonic measurement into a tissue bath containing 20 ml De Jalon solution. This solution was kept at 37 °C and gassed with 95% O_2, 5 % CO_2. The strips were subjected to a load of 2 g and allowed to stabilize for half an hour. Ketanserin (0.3 μM) and tropisetron (3 μM) were continuously present

in the De Jalon solution during all experiments to block contractile responses mediated by 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors. After stabilization, the longitudinal muscle was challenged with 0.3 μM methacholine. After washing and 10 min stabilization, the procedure was repeated in order to stabilize the response of the tissue.

Protocols

For the construction of concentration-response curves, drugs were applied directly to the tissue bath (added volume ≤ 1 % of bath volume). Methacholine (0.3 μM) was added to the bath in order to precontract the muscle.

5-HT. 10 Min after the addition of methacholine, the strips were first challenged with 0.3 μM 5-HT in the absence of an antagonist (ketanserin and tropisetron present). Then, 5-HT and methacholine were washed out by replacing the bathing fluid twice and stabilization of 10 min was allowed. After washout, this 20 min dosing cycle was repeated, applying 10 μM 5-HT. The relaxation to this latter administration of 5-HT was taken as 100 % relaxation. The size of the relaxation was measured as the maximum relaxation that was induced during an incubation period of approximately 2 min (at high concentrations in some cases up to 4 min). The compounds were washed out and again 10 min stabilization was allowed. Consecutively, drugs were administered, and were left with the preparations for 15 min. Then, non-cumulative concentration-response curves to 5-HT were established, applying the above described 20 min dosing cycle. Drugs, that were investigated for their possible effects against 5-HT-induced relaxations, were re-added directly after each washout. Of 4 segments that were taken from each animal, one strip was utilized as a control. The response to repetitive administration of methacholine was stable throughout the experiment. 5-HT (10 μM) induced relaxations of 5.2 ± 0.2 mm (n = 45).

ATP. The concentration-response curve to ATP was established in a similar non-cumulative dosing scheme as applied for 5-HT. Relaxations to ATP were expressed as a percentage of relaxations to ATP 3 μM, which induced relaxations of 3.3 ± 0.4 mm (n = 4).

Nitroprusside, nitroglycerin and isoprenaline. For the construction of concentration-response curves to nitroprusside, nitroglycerin and isoprenaline, the compounds were added in a cumulative fashion, 10 min after precontraction to methacholine-addition (0.3 μM). After washing and stabilization for 30 min

(washing every 10 min), a second concentration-response curve was established in the presence of cisapride. The responses induced by these agonists were expressed relatively to their maximal response during the first concentration-response curve for each segment. These maximal relaxations were respectively 12.4 ± 1.2 mm (nitroprusside 30 μM; n = 4), 6.1 ± 0.5 mm (nitroglycerin 10 μM; n = 4), and 11.1 ± 0.8 mm (isoprenaline 3 μM; n = 4).

Statistical analysis

For graphical representation means ± standard error of the mean (S.E.) were calculated. Differences between mean values were tested with one way analysis of variance using Dunnett's *t*-test (control versus treatment) or the Scheffé *F*-test (treatment versus treatment), when appropriate (Wallenstein *et al.*, 1980). The level of significance was set at $P < 0.05$.

Drugs

Cinitapride tartrate, clebopride tartrate (Almirall, Spain), dazopride fumarate, zacopride HCl (Robins Co., USA), renzapride (Beecham, Great Britain), metoclopramide HCl (Delagrange, France), cisapride, R 76 186 (cis-4-amino-5-chloro-N-[1-[4-[4-(dimethylamino)-1-piperidinyl]-4-oxobutyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide) (see Figure 8.1), SDZ 205-557 (2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino) ethyl ester), NAN-190 HCl (1-(2-methoxyphenyl)-4-[4-(2-phthalimido)-butyl] piperazine), spiperone, phentolamine HCl (Janssen Research Foundation, Belgium), methacholine HCl (E. Merck, Germany), suramin sodium (Rhone-Poulenc, France), 5-hydroxytryptamine creatinine sulphate, TTX (tetrodotoxin) (Serva, Germany), isoproterenol tartrate, L-NNA (N^G-nitro-L-arginine), disodium ATP (adenosine 5'-triphosphate), sodium nitroprusside (Janssen Chimica, Belgium), nitroglycerin (Federa, Belgium), paroxetine HCl (Ferrosan, Denmark), citalopram HBr (Lundbeck, Denmark), pargyline HCl (Abbott, USA), N-hexanoyl-5-hydroxytryptophyl-5-hydroxytryptophan amide (hex-5-HTP-DP) and 5-hydroxyindalpine (5-OHIP) maleate (gift M.D. Gershon) were added to the tissue bath solution in volumes ≤ 1% of the bath volume. Ketanserin tartrate and tropisetron (Janssen Research Foundation, Belgium) were continuously present in the De Jalon solution. All compounds were dissolved in distilled water, except for cisapride, SDZ 205-557, R 76 186, metoclopramide, spiperone and tropisetron, which were dissolved in distilled water acidified with tartaric acid in the stock solution (pH ≥ 3). The stock solution for isoproterenol and 5-HT contained 0.25 μM ascorbic acid. This vehicle had no effect on the strips. The stock solution of TTX was prepared in advance and kept

frozen (-30 °C). The benzamides induced small contractions per se, but this effect had disappeared by the time methacholine was added.

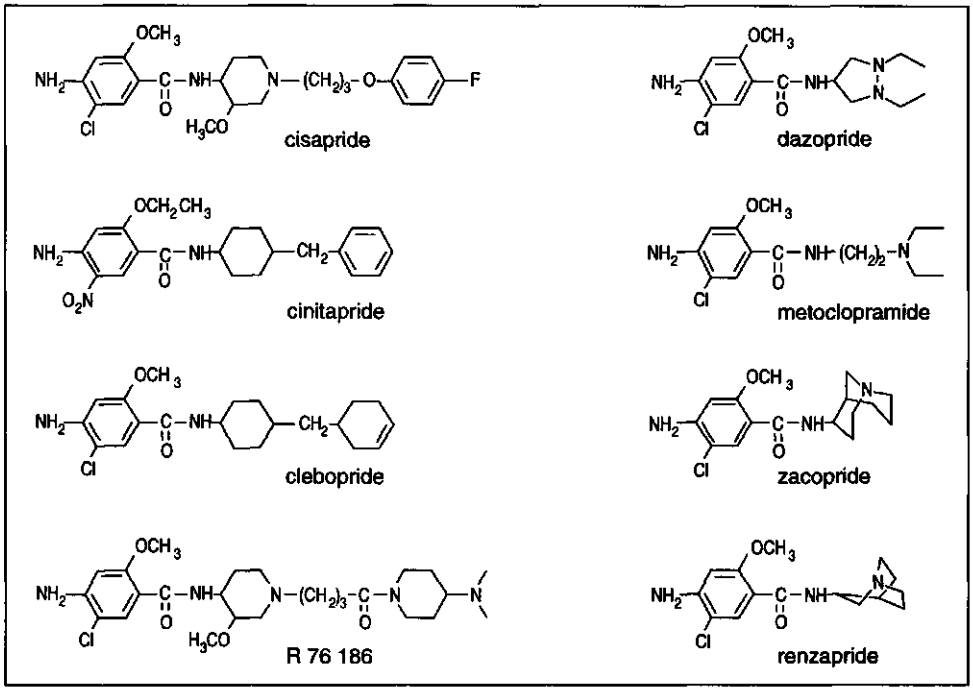


Figure 8.1 Structural formulas of the prokinetic benzamides that were tested.

Results

General

Over a concentration range of 0.03 μM to 100 μM, 5-HT induced relaxations in a concentration-dependent fashion (Figure 8.2, *upper panel*). The concentration-response curve to 5-HT was non-monophasic, with a first phase ranging from 0.03 μM to 1 μM, and a second phase from 1 μM to 30 μM, where the maximum effect to 5-HT was observed at 30 μM (Figure 8.2, *lower panel*).

Effects of cisapride and other substituted benzamides

From 0.1 μM onwards, cisapride significantly ($P < 0.05$) affected the concentration-response curve to 5-HT (Figure 8.2, *lower panel*). At a concentration of

0.1 μM, cisapride significantly enhanced the 5-HT-induced relaxations from 1 μM 5-HT onwards (i.e. the second phase of the concentration-response curve to 5-HT) by ca. 20-30 % (Figure 8.2, *lower panel*). At 0.3 and 1 μM, cisapride even further enhanced the relaxations to 5-HT (up to ca. 40 % amplification; Figure 8.2 *lower panel*). At these concentrations, cisapride also significantly inhibited the relaxations to 5-HT 0.1 μM. As can be seen from Figure 8.2 (*middle panel*), cisapride changed the time-relaxation profile, rendering it into a more pronounced and less tonic relaxation. At 3 μM cisapride merely inhibited the relaxations to 5-HT (up to 3 μM 5-HT) (Figure 8.2, *lower panel*).

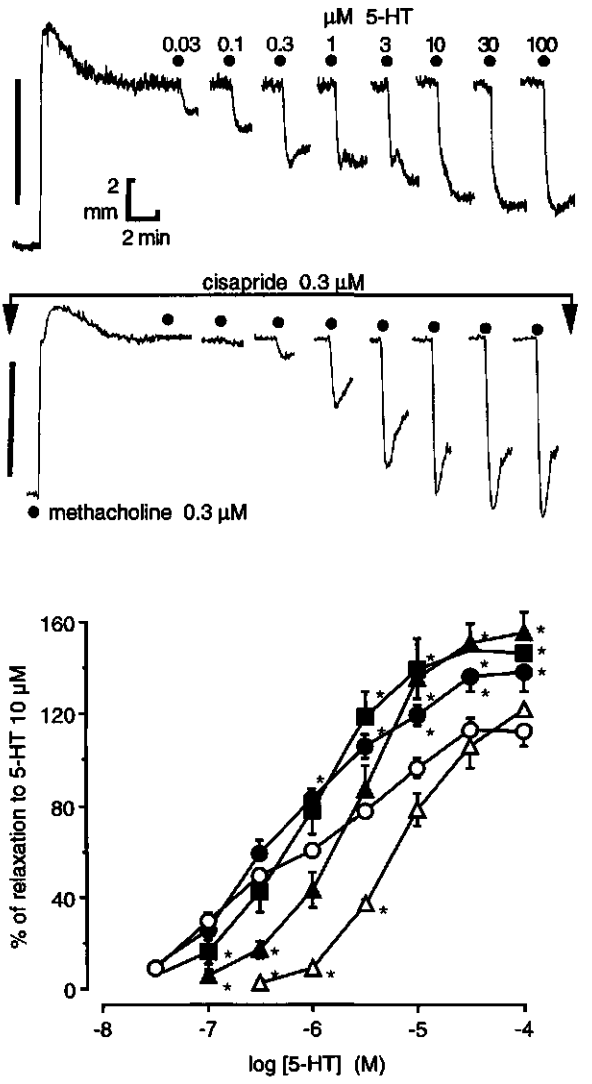


Figure 8.2 5-HT, administered in a non-cumulative fashion, induces relaxations on the methacholine-precontracted guinea pig proximal colon segments (*upper panel*). The experiments were done in the presence of ketanserin (0.3 μM) and tropisetron (3 μM) to block contractile responses to 5-HT mediated by 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors. The *middle panel* shows a recorder tracing of the effect of cisapride 0.3 μM on the 5-HT-induced relaxations. The vertical bars left to the tracing of the methacholine-induced precontraction represent the magnitude of the relaxation to 5-HT (10 μM) of that particular strip prior to the establishment of the concentration-response curve. In the *lower panel*, concentration-response curves to 5-HT in the absence (○) (n = 12), or presence of cisapride is shown: (●) 0.1 μM, (■) 0.3 μM, (▲) 1 μM, (△) 3 μM (all n = 6); means ± S.E. Relaxations were expressed as a percentage of a relaxation to 5-HT 10 μM in the absence of cisapride. Statistically significant difference from control is denoted with an asterisk.

Several other structural analogs of cisapride were tested against the 5-HT-induced relaxations (all at a concentration of 1 μM). Cinitapride, clebopride and dazopride showed effects that were very similar to those induced by cisapride: relaxations to lower concentrations (≤ 3 μM) of 5-HT were inhibited, while relaxations to higher concentrations (≥ 30 μM) of 5-HT were enhanced (up to ca. 40 %) (Figure 8.3, *left panel*). In contrast, renzapride only caused a slight enhancement of 5-HT-induced relaxations from 1 μM 5-HT onwards (up to ca. 20 %) (Figure 8.3, *right panel*). Zacopride only significantly ($P < 0.05$) enhanced the relaxations to 5--HT from 1 μM to 10 μM, while metoclopramide had no significant effect (Figure 8.3, *right panel*). R 76 186 did not significantly affect the concentration-response curve to 5-HT (n = 5; not shown).

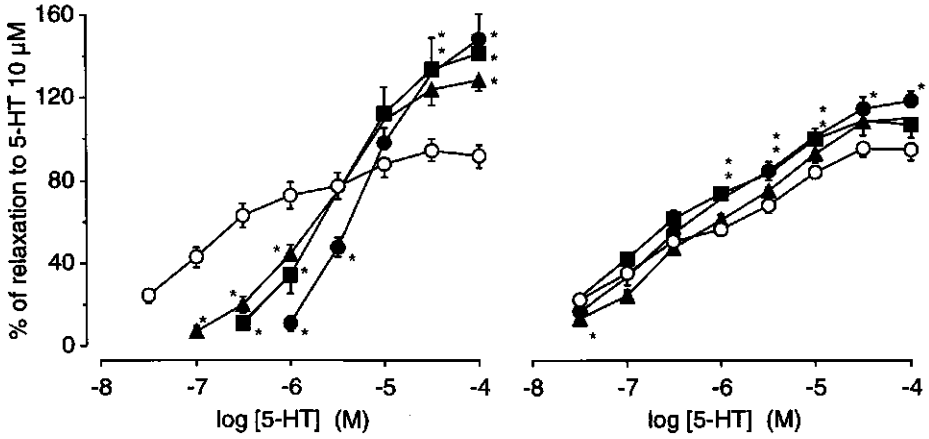


Figure 8.3 Concentration-response curves to 5-HT in the absence (○) or presence of several structural analogs (1 μM) of cisapride are shown: *left panel* cinitapride (●), clebopride (■) and dazopride (▲); *right panel* renzapride (●), zacopride (■) and metoclopramide (▲). All experiments n = 6; means ± S.E. The experiments were done in the presence of ketanserin (0.3 μM) and tropisetron (3 μM) to block contractile responses to 5-HT mediated by 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors. Relaxations were expressed as a percentage of a relaxation to 5-HT 10 μM in the absence of benzamide. Statistically significant difference from control is denoted with an asterisk.

In order to investigate the specificity of the cisapride-induced enhancement of 5-HT-induced relaxations, cisapride (0.3 μM) was tested against relaxations induced by ATP and isoproterenol, and by the NO donors nitroglycerin and nitroprusside. Cisapride did not significantly affect the concentration-response curves to either of these agonists (Figure 8.4).

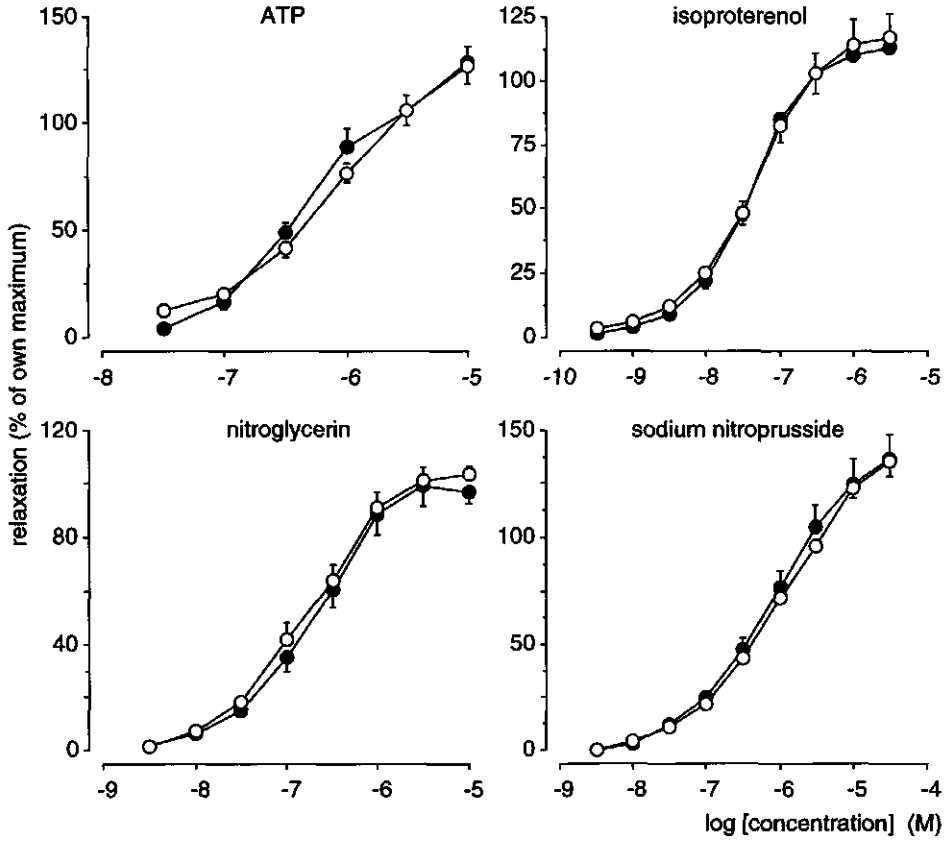


Figure 8.4 Concentration-response curves to ATP (*upper left panel*), isoproterenol (*upper right panel*), nitroglycerin (*lower left panel*) and sodium nitroprusside (*lower right panel*) in the absence (O) or presence (●) of cisapride (0.3 μM). All experiments n = 4; means ± S.E. The experiments were done in the presence of ketanserin (0.3 μM) and tropisetron (3 μM) to block contractile responses to 5-HT mediated by 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors. Relaxations were expressed as a percentage of a relaxation to respectively ATP (3 μM), isoproterenol (3 μM), nitroglycerin (10 μM) or nitroprusside (30 μM) in the absence of cisapride.

Effects of cisapride on nitrergic and purinergic neurotransmission

TTX (0.3 μM) abolished the relaxations to 5-HT in the presence of cisapride (1 μM) (n = 4; not shown). The NO synthase inhibitor L-NNA (100 μM) strongly inhibited the relaxations to 5-HT (Figure 8.5, *upper panel*), showing that the greater part of the relaxation to 5-HT is due to NO. In the presence of L-NNA, cisapride (0.3 μM) induced a 2-3 fold amplification of the remaining relaxation to 5-HT (Figure 8.5, *upper panel*). The purinoceptor antagonist suramin (300 μM) depressed the second, but not the first, phase of the curve to 5-HT (significant

from 10 μM 5-HT onwards) (Figure 8.5, *middle panel*). In the presence of suramin, cisapride (0.3 μM) did not have a significant effect on the curve to 5-HT, except for a small inhibition at 0.1 and 0.3 μM 5-HT (Figure 8.5, *middle panel*). In the presence of both cisapride and L-NNA, the remaining relaxations to 5-HT were virtually abolished by suramin (Figure 8.5, *lower panel*).

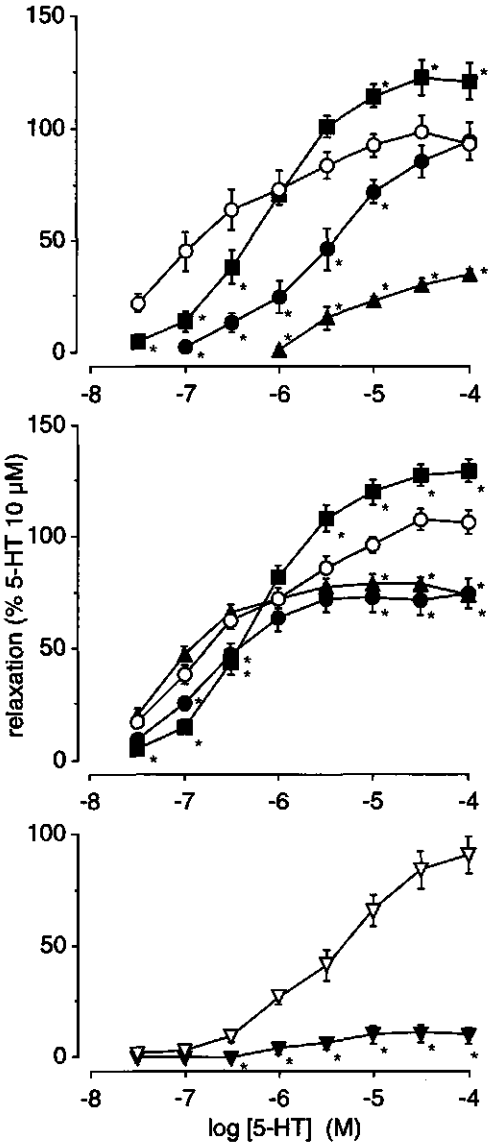


Figure 8.5 Concentration-response curves to 5-HT in the absence (O) or presence of several drugs: *upper panel* cisapride (0.3 μM; ■), L-NNA (100 μM; ▲) and the combination of cisapride and L-NNA (●) (n = 6); *middle panel* cisapride (0.3 μM; ■), suramin (300 μM; ▲), and the combination of cisapride and suramin (●) (n = 10); *lower panel* the combination of cisapride and L-NNA with (▼) or without (▽) suramin (n = 4); means ± S.E. The experiments were done in the presence of ketanserin (0.3 μM) and tropisetron (3 μM) to block contractile responses to 5-HT mediated by 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors. Relaxations were expressed as a percentage of a relaxation to 5-HT 10 μM in the absence of antagonist. Statistically significant difference from control is denoted with an asterisk.

Possible mechanisms

The 5-HT_{1P} receptor antagonist hex-5-HTP-DP (1 μM) had no intrinsic effect, nor did it affect the concentration-response curve to 5-HT (n = 4; not shown). The 5-HT_{1P} receptor agonist 5-OHIP (1 μM) also had no intrinsic effect, but it inhibited the first phase of the concentration-response curve to 5-HT (up to 1 μM), while the second phase was unaffected (n = 4; not shown). In the presence of 5-OHIP (1 μM), cisapride (0.3 μM) still enhanced the relaxations to 5-HT (10-100 μM) by about 25 %.

Cisapride might cause enhancement of the 5-HT-induced relaxations by inhibition of 5-HT uptake or 5-HT degradation by mono amine oxidase (MAO). The amplifying effects of cisapride on the concentration-response curve to 5-HT were however not mimicked by the specific 5-HT uptake inhibitors citalopram and paroxetine (0.3 μM; n = 5) (not shown), or by the MAO inhibitor pargyline (100 μM; n = 4) (not shown). As cisapride has moderate affinity for α-adrenoceptors, it was investigated whether blockade of α-adrenoceptors could explain the effects of cisapride. The non-selective α-adrenoceptor antagonist phentolamine (1 μM) did not significantly affect the relaxations to 5-HT (n = 5; not shown). Antagonism of transmitter release-inhibiting 5-HT_{1A} receptors might account for the effects of cisapride. The 5-HT_{1A} receptor antagonists NAN-190 (0.03 μM) and spiperone (1 μM) did not amplify the relaxations to 5-HT, but spiperone inhibited them from 10 μM 5-HT onwards (n = 6; not shown). It is conceivable that the presence of 3 μM tropisetron in the antagonist cocktail is insufficient to block all of the 5-HT_4 receptor-mediated contraction, and that desensitization of these receptors by cisapride explains the enhancing effects of cisapride on the 5-HT-induced relaxations (i.e. elimination of functional antagonism). However, the 5-HT_4 receptor antagonist SDZ 205-557 (0.3 μM) did not mimick the effects of cisapride (n = 4; not shown).

Discussion

Previously, we have shown that the 5-HT-induced relaxations on the guinea pig colon are mediated by nitric oxide (Briejer *et al.*, 1992) and ATP (Briejer *et al.*, 1995). It was shown, that suramin (and apamin) strongly inhibited relaxations to exogenous ATP, but did not affect those induced by nitroglycerin, exogenous NO and isoproterenol (Briejer *et al.*, 1995). However, both in the previous and current study, suramin selectively depressed the second, but not the first, phase of the concentration-response curve to 5-HT. ATP is an established (co-)trans-

mitter, and suramin is known to be a selective antagonist of ATP-induced P_2-purinoceptor mediated responses (Burnstock, 1972; Burnstock & Kennedy, 1985; Kennedy, 1990). Hence, it was concluded that ATP might be a mediator of 5-HT-induced relaxations in this preparation. This second phase is selectively amplified by cisapride and some structural analogs, and these amplified relaxations can be inhibited by suramin, but not by the NO synthase inhibitor L-NNA. Furthermore, both in the absence and presence of cisapride, relaxations were abolished by the neurotoxin TTX (Briejer *et al.*, 1992; this study), and cisapride did not affect relaxations evoked by exogenous ATP or the NO donors nitroglycerin or nitroprusside. These observations suggest, that cisapride selectively enhances purinergic neurotransmission through a prejunctional mode of action.

As the effects of cisapride on relaxations to 5-HT were mimicked by its structural analogs cinitapride, dazopride, clebopride and to a lesser extent renzapride and zacopride (see Figure 8.1), it is suggested that these benzamides have a similar mode of action. The 5-HT_4 receptor agonist R 76 186 (Briejer *et al.*, 1993) and metoclopramide did not significantly affect the concentration-response curve to 5-HT. Apparently, the 2-methoxy-4-amino-5-chloro-substituted benzamide moiety is not sufficient to elicit the amplification effect, as it is present in all benzamides that were tested. Cinitapride, containing a 2-ethoxy-4-amino-5-chloro-substituted benzamide moiety instead (see Figure 8.1), was about equally effective to cisapride in affecting the relaxations to 5-HT. Hence, a qualitative structure-activity relationship is not obvious.

The concentration-response curve to 5-HT is non-monophasic, which suggests that two different 5-HT receptors are involved. The first phase is completely inhibited by the NO synthase inhibitor L-NNA (Briejer *et al.*, 1992; current results), and the second phase is inhibited by suramin (Briejer *et al.*, 1995; current results). Hence, it is proposed that the guinea pig colon is endowed with a high affinity receptor (5-HT_H), causing the generation of nitric oxide, and a low affinity receptor (5-HT_L) whose stimulation induces the release of ATP. In another study, we have also found several lines of evidence for such a two-receptor system (Briejer *et al.*, submitted). The selective inhibition of spiperone of only the second phase of the concentration-response curve to 5-HT is in good agreement with the assumption that two 5-HT receptors are involved.

In previous studies, the 5-HT-induced relaxations were shown to be inhibited by 5-HT_1 receptor antagonists, but did not resemble one of the currently known 5-HT_1 receptor subtypes (Kojima, 1991; Elswood & Bunce, 1992; Briejer *et al.*, 1992). As cisapride, and some of the other benzamides tested, shifted the first phase of the concentration-response curve to 5-HT to the right, it is likely that

these drugs are antagonists of the 5-HT_H receptor. This shift of the curve to the right probably explaines the apparent lack of enhancement of the 5-HT-induced relaxations at 3 μM cisapride. Cisapride is known to have affinity for 5-HT_{1A}, 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors (Van Nueten & Schuurkes, 1989; Leysen, 1990; Taniyama *et al.*, 1991; Briejer *et al.*, 1993), and interferes with putative 5-HT_{1P} receptor-mediated responses (Nemeth *et al.*, 1985). Our data suggest that it also interacts with the putative 5-HT_H receptor.

Several mechanisms can be proposed by which cisapride (and some of the other above described benzamides) could enhance relaxations to 5-HT. As cisapride seems to act prejunctionally, and the purinoceptor-mediated part of the response to 5-HT is selectively amplified, it is likely that the amount of released ATP is increased by cisapride, though only direct measurement of the amount of released ATP would allow a definite conclusion on this.

Prejunctional α_2-adrenoceptors and 5-HT_{1A} receptors are known to exist in the enteric nervous system, and both receptor types are known to inhibit transmitter release after stimulation (Kojima *et al.*, 1988; Galligan, 1993). Leakage of noradrenaline from enteric neurons might continuously stimulate the prejunctional α_2-adrenoceptors, thus inhibiting ATP release. In binding experiments, however, cisapride has moderate affinity for α_1-adrenoceptors, but not for α_2-adrenoceptors (J.E. Leysen, personal communication). Furthermore, the non-selective α-adrenoceptor antagonist phentolamine did not mimick the effects of cisapride. Hence, an action at α_2-adrenoceptors cannot account for the amplifying effects of cisapride.

In electrophysiological experiments with guinea pig enteric neurons, 5-HT and cisapride are known to induce hyperpolarizations in some neurons, which are thought to be caused by stimulation of 5-HT_{1A} receptors (Nemeth *et al.*, 1985). In functional experiments with preparations of the guinea-pig ileum, 5-HT and its analog 5-carboxamidotryptamine have been shown to inhibit field stimulation-induced acetylcholine release (Galligan, 1992). These effects could be inhibited by NAN-190, a novel selective 5-HT_{1A} receptor antagonist, and spiperone. From similar experiments, other investigators have reported that cisapride inhibited 5-HT_{1A} receptor-mediated inhibition of acetylcholine release (Taniyama *et al.*, 1991). In binding experiments, the affinity of cisapride for 5-HT_{1A} receptors (cloned human 5-HT_{1A} Ha-7 cells, 8-hydroxy-2-(di-N-propylamino) tetralin displacement) was 6.1 (J.E. Leysen, personal communication). It is known that partial agonists behave as antagonists, when applied together with a full agonist at a common receptor, for the partial agonist prevents maximal stimulation of the common receptor by the full agonist (see:

Hoyer & Boddeke, 1993). If it is supposed that 5-HT_{1A} receptors are also present on the inhibitory purinergic neurons and that cisapride is a partial agonist of 5-HT_{1A} receptors, apparent antagonist effects of cisapride would thus explain the enhancement of the ATP release. However, spiperone and NAN-190, at concentrations that inhibited 5-HT_{1A} receptor-mediated effects in the guinea pig ileum (see above; Galligan, 1992), failed to mimick the effects of cisapride. Furthermore, to date 5-HT_{1A} receptors have been found to be present exclusively on excitatory neurons (seen above). Hence, a 5-HT_{1A} receptor mechanism is also not likely to account for the amplifying effects of cisapride.

In electrophysiological experiments with a subset of type 2/AH myenteric neurons of the guinea pig, slow prolonged depolarizations can be elicited by 5-HT, that are not sensitive to classical 5-HT receptor antagonists (Nemeth *et al.*, 1985; Takaki *et al.*, 1985). The receptor that mediates this effect, has provisionally been named 5-HT_{1P}, though it does not belong to the 5-HT_1-like group of receptors as defined by Humphrey *et al.* (1993). The 5-HT-induced depolarizations can be mimicked by 5-OHIP and the S-enantiomer of zacopride (Mawe *et al.*, 1986; Wade *et al.*, 1991), and inhibited by a dipeptide of 5-hydroxytryptophan, i.e. hex-5-HTP-DP (Takaki *et al.*, 1985), and the benzamides R-zacopride, renzapride and cisapride (Nemeth *et al.*, 1985; Mawe *et al.*, 1989; Wade *et al.*, 1991). If cisapride would slightly depolarize the ATP-containing neurons, it is conceivable that the generation of action potentials is facilitated, and thus more ATP is released. However, cisapride is an antagonist of 5-HT_{1P} receptor-mediated depolarizations, and therefore it is difficult to explain the amplifying effects of cisapride and some of the other benzamides tested in terms of interactions with 5-HT_{1P} receptors. Furthermore, the 5-HT_{1P} receptor antagonists hex-5-HTP-DP and renzapride had no effect on the relaxations to 5-HT, and the agonist 5-OHIP merely depressed the first phase of the concentration-response curve to 5-HT, while leaving the second phase unaffected. The structural formula of 5-OHIP resembles that of 5-HT, and therefore, it is not surprising that 5-OHIP acts as an antagonist at 5-HT_H receptors. From these observations it can be concluded that 5-HT_{1P} receptors are not likely to be involved in the 5-HT-relaxation-enhancing effects of cisapride.

Inhibition of 5-HT degradation by MAO, or inhibition of 5-HT uptake by the neurons, might cause enhancement of the effects of 5-HT. However, inhibition of MAO or 5-HT uptake did not mimick the effects of cisapride. Furthermore, inhibition of either removal system would be expected to cause a shift of the concentration-response curve to the left, and not, as in our experiments, a selective enhancement of the responses. Hence, also inhibition of 5-HT uptake or MAO do not explain the effects of cisapride.

The substituted benzamides are agonists at the 5-HT_4 receptor, whose stimulation causes contraction of the guinea-pig gut. These contractions are prone to desensitization (Bockaert *et al.*, 1992; Briejer *et al.*, 1993; Ford & Clarke, 1993). The 5-HT_4 receptors might be incompletely blocked by tropisetron (pA_2 = 6.0-6.7; see Bockaert *et al.*, 1992; Ford & Clarke, 1993) in our experiments. Desensitization of a part of the relaxation-counteracting 5-HT_4 receptors that is not blocked by tropisetron, could therefore result in an apparent enhancement of the relaxations to 5-HT. Therefore, the selective 5-HT_4 receptor antagonist SDZ 205-557 (Buchheit *et al.*, 1992; see: Ford & Clarke, 1993) was tested in addition to tropisetron, but it did not mimick the effects of cisapride. Furthermore, if 5-HT_4 receptor desensitization were the mechanism of action, 5-HT_4 receptor agonists like R 76 186 (Briejer *et al.*, 1993), renzapride and zacopride (Bockaert *et al.*, 1992) would also enhance the relaxations to 5-HT, but these compounds had no or only a weak effect. Hence, 5-HT_4 receptors do not seem to be involved.

Propulsion of gut contents is established by a coordinated pattern of contractions and relaxations of both the longitudinal and circular smooth muscle coat in the gut wall. The stimulation (prokinetic) effects of cisapride in both animal and man has been explained in terms of facilitation of cholinergic and non-cholinergic neurotransmission which favours contraction during peristalsis (Schuurkes *et al.*, 1985; Tonini *et al.*, 1991; Taniyama *et al.*, 1991; Kilbinger & Wolf, 1992; Bingham & Andrews, 1992). The current results suggest that cisapride (and some other prokinetic benzamides) can also facilitate inhibitory neurotransmission, which could favour relaxation during peristalsis and could thus contribute to its prokinetic effects. Future research will be needed to evaluate whether these findings are relevant for the clinical efficacy of cisapride.

Conclusions: cisapride, and some of its structural analogues, seem to selectively enhance the suramin-sensitive, purinoceptor-mediated, part of the neurogenic relaxations to 5-HT. Though it was shown that cisapride acts presynaptically, it is not known by what mechanism it amplifies the relaxations to 5-HT.

Acknowledgements

Dr. M.D. Gershon is acknowledged for the donation of hex-5-HTP-DP and 5-OHIP.

References

Beani L, Bianchi C, Crema C (1970) Effects of metoclopramide on isolated guinea-pig colon. 1) Peripheral sensitization to acetylcholine. *Eur J Pharmacol* 12: 320-331.

Bingham S, Andrews PLR (1992) Gastrointestinal motility effects of cisapride mediated by non-adrenergic, noncholinergic nerves. In: *Gastrointestinal dysmotility: focus on cisapride* (Heading RC, Wood JC Eds.) Raven Press Ltd., New York, pp. 93-106.

Bockaert J, Fozard JR, Dumuis A, Clarke DE (1992) The 5-HT_4 receptor: a place in the sun. *Trends Pharmacol Sci* 13: 141-145.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1992) Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*. *Br J Pharmacol* 107: 756-761.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1993) Cisapride and a structural analogue, R 76 186, are 5-hydroxytryptamine$_4$ (5-HT_4) receptor agonists on the guinea-pig colon ascendens. *Naunyn-Schmiedeberg's Arch Pharmacol* 347: 464-470.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1995) 5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide. *Naunyn-Schmiedeberg's Arch Pharmacol* 351: in press.

Buchheit KH, Gamse R, Pfannkuche HJ (1992) SDZ 205-557, a selective, surmountable antagonist at 5-HT_4 receptors in the isolated guinea-pig ileum. *Naunyn-Schmiedeberg's Arch Pharmacol* 345: 387-393.

Burleigh DE, Trout SJ (1985) Evidence against an acetylcholine releasing action of cisapride in the human colon. *Br J Clin Pharmacol* 20: 475-478.

Burnstock G (1972) Purinergic nerves. *Pharmacol Rev* 24: 509-581.

Burnstock G, Kennedy C (1985) Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen Pharmacol* 16: 433-440.

De Ridder WE, Schuurkes JAJ (1993) Cisapride and 5-hydroxytryptamine enhance motility in the canine antrum *via* separate pathways, not involving 5-hydroxytryptamine$_{1,2,3,4}$ receptors. *J Pharmacol Exp Ther* 264: 79-88.

Elswood CJ, Bunce KT (1992) Investigation of the 5-HT receptor mediating relaxation in guinea-pig proximal colon. *J Pharm Pharmacol* 44: 264-266.

Ford APDW, Clarke DE (1993) The 5-HT_4 receptor. *Med Res Rev* 13: 633-662.

Galligan JJ (1992) Differential inhibition of cholinergic and noncholinergic neurogenic contractions by 5-hydroxytryptamine$_{1A}$ receptor agonists in guinea pig ileum. *J Pharmacol Exp Ther* 260: 306-312.

Galligan JJ (1993) Differential inhibition of cholinergic and noncholinergic neurogenic contractions by mu opioid and alpha-2 adrenergic agonists in guinea pig ileum. *J Pharmacol Exp Ther* 264: 375-383.

Gullikson GW, Virina MA, Loeffler RF, Yang DC, Goldstin B, Flynn DL, Moormann AE (1992) Gastrointestinal motility responses to the S and R enantiomers of zacopride, a 5-HT_4 agonist and 5-HT_3 antagonist. *Drug Dev Res* 26: 405-417.

Hoyer D, Boddeke HWGM (1993) Partial agonists, full agonists, antagonists: dilemmas of definition. *Trends Pharmacol Sci* 14: 270-275.

Humphrey PPA, Hartig P, Hoyer D (1993) A proposed new nomenclature for 5-HT receptors. *Trends Pharmacol Sci* 14: 223-236.

Kennedy C (1990) P_1- and P_2-purinoceptor subtypes - an update. *Arch Int Pharmacodyn* 303: 30-50.

Kilbinger H, Wolf, D (1992) Effects of 5-HT_4 receptor stimulation on the basal and electrically evoked release of acetylcholine from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch Pharmacol* 345: 270-275.

Kojima S (1991) Characterization of the 5-hydroxytryptamine-induced relaxations of the guinea-pig proximal colon. *Arch Int Pharmacodyn* 313: 23-32.

Kojima S, Sakato M, Shimo Y (1988) An α_2-adrenoceptor-mediated inhibition of non-adrenergic non-cholinergic inhibitory responses of the isolated proximal colon of the guinea-pig. *Asia Pacific J Pharmacol* 3: 69-75.

Leysen JE (1990) Gaps and peculiarities in 5-HT_2 receptor studies. *Neuropsychopharmacology* 3: 361-396.

Massingham R, Bou J, Roberts DJ (1985) A comparison of the stimulatory effects of metoclopramide and cinitapride in the guinea-pig isolated ileum. *J Auton Pharmacol* 5: 41-53.

Mawe GM, Branckek TA, Gershon MD (1986) Peripheral neural serotonin receptors: identification and characterization with specific antagonists and agonists. *Proc Natl Acad Sci USA* 83: 9799-9803.

Mawe GM, Branchek TA, Gershon MD (1989) Blockade of 5-HT-mediated enteric slow EPSPs by BRL 24924: gastrokinetic effects. *Am J Physiol* 257: G386-G396.

Müller-Lissner SA (1987) Treatment of chronic constipation with cisapride and placebo. *Gut* 28: 1033-1038.

Nemeth PR, Ort CA, Zafirov DH, Wood JD (1985) Interactions between serotonin and cisapride on myenteric neurons. *Eur J Pharmacol* 108: 77-83.

Pinder RM, Brogden RN, Sawyer PR, Speight TM, Avery GS (1976) Metoclopramide: a review of its pharmacological properties and clinical use. *Drugs* 12: 81-131.

Reynolds JC, Putnam PE (1992) Prokinetic agents. *Gastroenterol Clin North Am* 21: 567-596.

Reyntjens A, Verlinden M, Aerts T (1986) Development and clinical use of the new gastrointestinal prokinetic drug cisapride (R 51 619). *Drug Dev Res* 8: 251-265.

Schuurkes JAJ, Van Nueten JM, Van Daele PGH, Reyntjens AJ, Janssen PAJ (1985) Motor stimulating properties of cisapride on isolated gastrointestinal preparations of the guinea-pig. *J Pharmacol Exp Ther* 234: 775-783.

Takaki M, Branchek T, Tamir H, Gershon MD (1985) Specific antagonism of enteric neural serotonin receptors by dipeptides of 5-hydroxytryptophan: evidence that serotonin is a mediator of slow synaptic excitation in the myenteric plexus. *J Neurosci* 5: 1769-1780.

Taniyama K, Nakayama S, Takeda K, Matsuyama S, Shirakawa J, Sano I, Tanaka C (1991) Cisapride stimulates motility of the intestine *via* the 5-hydroxytryptamine receptors. *J Pharmacol Exp Ther* 258: 1098-1104.

Tonini M, Rizzi CA, Manzo L, Onori L (1991) Novel enteric 5-HT_4 receptors and gastro-intestinal prokinetic action. *Pharmacol Res* 24: 5-14.

Van Nueten JM, Schuurkes JAJ (1989) 5-HT_3 serotonergic mechanisms and stimulation of gastro-intestinal (GI) motility by cisapride. *Br J Pharmacol* 96 suppl: 331P.

Wade PR, Mawe GM, Branchek TA, Gershon MD (1991) Use of stereoisomers of zacopride to analyze actions of 5-hydroxytryptamine on enteric neurons. *Am J Physiol* 260: G80-G90.

Wallenstein S, Zucker CL, Fleiss JL (1980) Some statistical methods useful in circulation research. *Circ Res* 47: 1-9.

Chapter 9

A novel *in vivo* method for the assessment of peristalsis in the distal colon of the guinea-pig

M.R. Briejer, M. Tonini, L.M.A. Akkermans & J.A.J. Schuurkes

submitted for publication

Abstract. A novel method for the study of colonic peristalsis in the anaesthetized guinea-pig was developed. After laparotomy, a rubber balloon was inserted into the lumen of the distal colon and inflated with 0.05-0.2 ml of water to mimic faecal pellet size. Thus, movement of the balloon and change in length of the longitudinal muscle could be assessed by isotonic recording.

Inflation of the balloon per se did not induce propulsion of the balloon. Treatment with the α-adrenoceptor antagonist phentolamine (2 mg/kg i.v.) induced peristalsis and balloon displacement. This suggests that sympathetic nerve over-activity, probably due to anaesthesia and surgery, exerts a tonic inhibitory influence on the colon, which prevents coordinated motor activity. The opioid antagonist naloxone (1 mg/kg i.v.) did not promote peristalsis when given alone. However, when naloxone was administered in addition to phentolamine, a decrease in the maximum, but not the average velocity of propulsion was observed compared to treatment with phentolamine alone. Treatment with the NO synthase inhibitor N^G-nitro-L-arginine (25 mg/kg i.v.) in addition to phentolamine increased the average, but not the maximum, pellet propulsion velocity as compared to treatment with phentolamine alone. Irrespective of the treatment schedule employed, the velocity of propulsion was not dependent upon the inflation volume of the balloon.

This model provides the first *in vivo* method for the investigation of drugs to induce or affect colonic propulsion.

Introduction

The intrinsic enteric nervous system of the gut, which controls its motility, is considered the third part of the autonomic nervous system (see: Gershon & Erde, 1981). Sympathetic (inhibitory) and parasympathetic (both excitatory and inhibitory) inputs from extrinsic nerves merely modulate the intestinal motor activity. Many functional *in vitro* models have been described to investigate the pharmacological modulation of gastrointestinal motility. Such models usually comprise isolated parts of the guinea-pig gastrointestinal tract, in which parameters like force, pressure, electrical properties or pellet propulsion are assessed in response to distension, pressure, or electrical or pharmacological stimulation (for example: Trendelenburg, 1917; Frigo & Lecchini, 1970; Costa & Furness, 1976). In such preparations, extrinsic parasympathetic and sympathetic nerve input (and output, feedback loops), as well as blood supply, have usually been cut off together with the mesentery. *In vivo* models differ in this respect, though in anaesthetized animals, anaesthesics and abdominal surgery influence sympathetic and parasympathetic tone (and possibly also other neurotransmitter balances, e.g. opioid peptides), and thus affect gastrointestinal motility (see: Dubois, 1988; Galligan *et al.*, 1986). Here we describe a novel *in vivo* model in the anaesthetized guinea-pig, which allows measurement of the velocity of propulsion of an inflated balloon in the distal colon, and the effects of drugs thereon.

Methods

Preparation of the animals

Dunkin-Hartley guinea-pigs of both sexes, weighing 700-800 g, were used that had access to food and water *ad libitum*. The guinea-pigs (12 in total) were anaesthetized with a mixture of $N_2O : O_2$ in a ratio of 2 : 1 and halothane (Fluothane®, 3-3.5 % induction, 1-1.5 % maintenance). The animals breathed the gas mixture spontaneously by means of a cap positioned over the head. The abdomen and throat were shaved, and the animals were mounted upon a heating pad maintained at 38 °C. The left carotid artery and the right external jugular vein were cannulated for monitoring blood pressure and to allow the i.v. administration of drugs, respectively. Heart rate was also monitored by means of 4 electrodes that were attached subcutaneously.

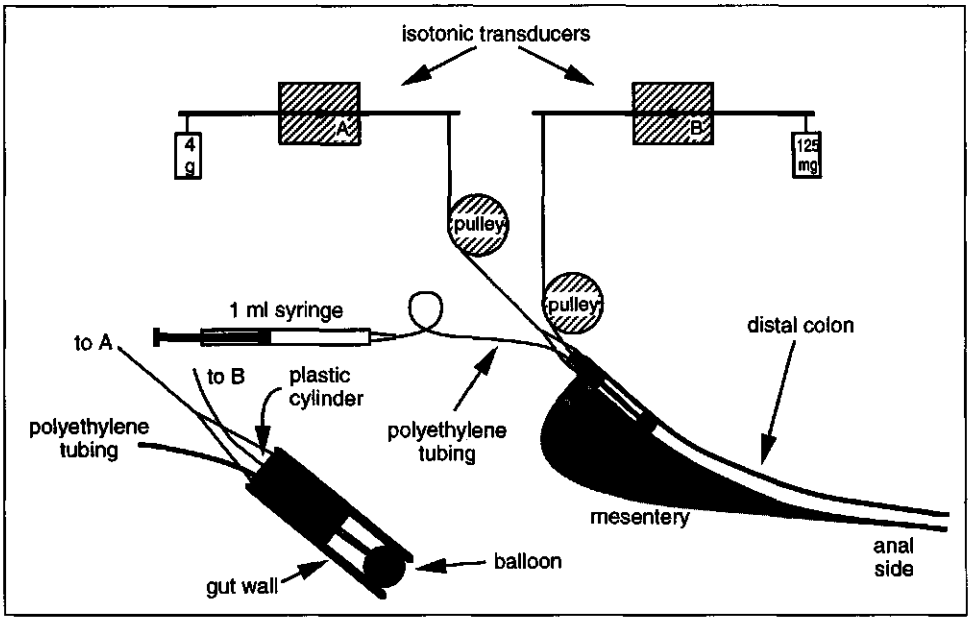


Figure 9.1 Schematic drawing of the experimental set-up. After laparotomy of the anaesthetized guinea-pig, the distal part of the colon was cut and fixed in a lifted position by insertion of a small plastic cylinder, that was attached to an isotonic transducer (transducer A, 4 g load) via a pulley. A second isotonic transducer (transducer B, 125 mg load) was connected to a small balloon, that could be inserted into the lumen of the distal colon, and that could be inflated by means of a syringe filled with water via a thin polyethylene tubing (see also magnified insert in the left lower corner for details). After inflation, length changes and propulsion velocity could thus be assessed simultaneously.

The abdominal cavity was exposed by a midline incision. The abdominal wall was lifted by means of stitches that were attached to two metal rods above the animal. These rods and stitches were positioned in such a manner, that the wound attenuated to a rectangular shape and an enlarged abdominal cavity. In case the caecum was very large due to faecal contents and/or gas, it was removed (8 guinea-pigs). In female animals, the intact uterus was fixated in a lifted position to avoid interference with the balloon movement. The distal part of the colon was lifted, and at about 10 cm proximal to the anus the colon was ligated, occluding the lumen. Under normal conditions, the distal colon serves to transport faecal pellets into the anal direction. About 10 cm of colon proximal to this ligature was removed after ligation of the supplying blood vessels, to avoid sterical hindrance. A few mm distal to the ligature, a small hole was made, and the luminal contents were washed out through the anus by gentle injection of prewarmed (38 °C) saline into the distal part of the colon. Then, a small plastic cylinder (approximately 1.3 cm long, outer diameter ca. 5 mm) was inserted through the hole and fixated. This plastic cylinder was attached to an isotonic transducer (load 4 g) via a pulley to detect changes in length. It was positioned just equally high to the wound borders with the aid of the pulley. A small balloon was prepared from a piece of condom by folding it over a thin polyethylene tubing and tiing it down with a thread. This thread was attached to a second isotonic transducer (load 125 mg) via a pulley, and the empty balloon was inserted through the plastic cylinder into the lumen. The balloon could be inflated by injecting water through the polyethylene tubing by means of a syringe. Movement of the balloon at different diameters and change in length of the colon could thus be assessed simultaneously. The set-up as described here is schematically represented in Figure 9.1. During the experiments, care was taken that the abdominal cavity and its contents were kept moist, by regular spraying of prewarmed saline into the wound. To help maintain a constant temperature at the ventral side, the animals were placed under an infrared heating lamp, but the abdominal cavity was protected from radiation with a moist cotton wool swab.

Experimental protocol

The deflated balloon was inserted about 3 to 4 cm from the plastic cannula, and inflated to a volume of 0.05, 0.1, 0.15 or 0.2 ml, in a random order. Phentolamine and/or naloxone were injected through the venous cannula, about 3-5 min after the balloon had been inserted and inflated for the first time. After peristaltic propulsion had occurred, the balloon was deflated and gently

pulled back into the plastic cannula. After a resting period of 0.5-1 min, the balloon was reinserted and inflated, again to a randomly chosen volume, and the velocity of propulsion was determined. The number of peristaltic propulsion movements that were thus assessed, ranged from 4 to 18 per animal. Simultaneously, changes in length of the longitudinal coat were monitored.

Statistics and graphical representation

The average and maximum velocities were determined graphically. The average velocity during a peristaltic movement was defined as the distance of travelling divided by the time, whereby a peristaltic movement consisted of a period of continuous movement with no stop (≤ 1 mm movement in 5 s). The maximum velocity was determined from the maximum slope of the tracing. Velocities were averaged per animal for each of the volumes tested. N denotes the number of animals. Differences in mean values were compared with ANOVA and the Scheffé *F*-test for multiple comparisons. The level at which a difference was considered statistically significant was set at $P < 0.05$. For graphical representation, the mean ± standard error of the mean was calculated.

Drugs

The following drugs were used: naloxone HCl, phentolamine HCl (Regitine®), guanethidine, N^G-nitro-L-arginine (Janssen Chimica, Belgium). The drugs were dissolved in saline.

Giuliani *et al.* (1993) reported that treatment with the α-adrenoceptor antagonist phentolamine and the opioid receptor antagonist naloxone of anaesthetized guinea-pigs resulted in a higher reproducibility in the assessment of colonic motor reflexes. We therefore evaluated the effects of these drugs.

Results

No drug treatment

In most cases, the longitudinal coat showed only little activity as reflected by the constant base line (Figure 9.2), though in others, rithmic activity was recorded. Insertion and subsequent inflation of the balloon induced in some cases a small contraction of the longitudinal coat, and sometimes the rhythmic activity of the longitudinal coat increased, but neither of these effects was consistently observed. The balloon was not propelled under these circumstances, or

only over a small distance (< 1 cm). In the absence of any drug treatment, continuous peristaltic movements were never observed.

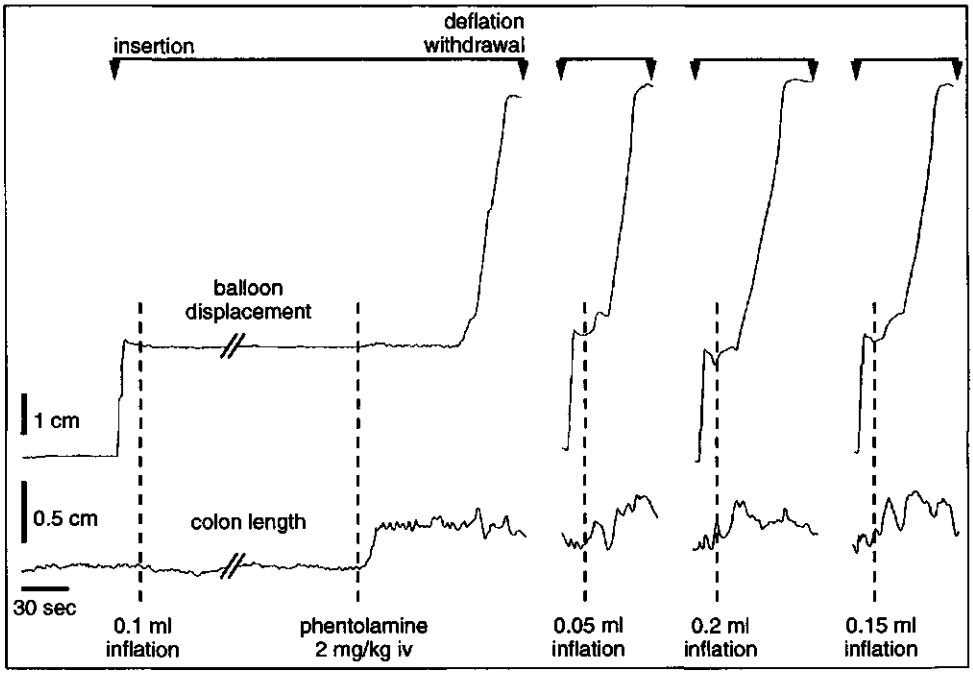


Figure 9.2 Recorder tracing of a typical experiment. In the upper tracing, displacement of the balloon is shown, while the lower tracing represents longitudinal length changes of the colon. As can be seen from this tracing, spontaneous movements were usually small in amplitude, though this varied in the different animals studied. Insertion of the balloon and inflating to a randomly chosen volume (in this case 0.1 ml) did usually not evoke any response. After 5 min, the α-adrenoceptor antagonist phentolamine 2 mg/kg was injected through the right jugular vein. Immediately after this, the longitudinal coat contracted, and the spontaneous movements increased in amplitude. About 1 min after injection, the pellet was propelled into the anal direction. After deflation and gentle withdrawal of the empty balloon into the plastic cylinder, the balloon could be reinserted (ca. 1 min rest) and inflated to a different volume.

Treatment with phentolamine

Intravenous injection of phentolamine (2 mg/kg) caused an immediate fall in blood pressure and a reflex tachycardia, which remained stable for at least 40 min (which was the maximum period of peristalsis assessment). Within 1 min after injection, a contraction and an increase in the spontaneous activity of the longitudinal coat were induced, and within 2 min after injection (after 4 min

in 1 case), the balloon was propelled (Figure 9.2). During or previous to a peristaltic wave, the longitudinal coat contracted, but, even within the same animal, this was often not observed (Figure 9.2). Sham injections with saline had no effect (n = 4). In some cases, one or two pellets were expelled upon administration of phentolamine, which apparently were still present in the distal part of the colon. There was no relationship between the distension of the balloon and the velocity of propulsion (Figure 9.3a+b), as the means of neither the average nor the maximum velocities at the different inflation volumes significantly differed from one another. The maximum velocity (which in most cases occurred in the last 2 or 3 cm) obtained was 4.57 ± 0.04 mm/s (n = 6; 0.1 ml inflation) and the maximum average velocity was 2.18 ± 0.12 mm/s (n = 6; 0.05 ml inflation). The lag time between inflation and movement varied from a few seconds (Figure 9.2) to a few minutes. In some cases, the peristaltic propulsion was already triggered by the insertion of the empty balloon, and therefore, it was difficult to determine a lag time.

Treatment with phentolamine and naloxone

Animals treated with both phentolamine (2 mg/kg) and naloxone (1 mg/kg) responded similarly to phentolamine-treated animals, though the combination treatment significantly decreased maximum velocity of propulsion at 0.15 and 0.2 ml (Figure 9.3b), while it tended to at 0.05 ml and 0.1 ml. The combination treatment with naloxone also tended to lower the average velocity of propulsion, though this was never statistically significant (Figure 9.3a). It was often difficult to insert the empty balloon into the lumen, and to pull it back after a peristaltic movement, which seemed to be caused by the high tone of the circular muscle. This was not observed if naloxone had not been administered. Hence, the decrease in maximum propulsion velocity of the balloon in the naloxone treated animals might reflect an increase in resistance due to high circular tone. Treatment with naloxone *per se* did not induce propulsion of the balloon (n = 3).

Treatment with phentolamine and N^G-nitro-L-arginine

In guinea-pigs treated with both phentolamine and the NO synthase inhibitor N^G-nitro-L-arginine (L-NNA; 25 mg/kg i.v.) average velocity of propulsion was increased at 0.05 ml, 0.10 ml and 0.15 ml inflation, but not at 0.20 ml inflation of the balloon (n = 3) (Figure 9.3b), as compared to treatment with phentolamine alone. However, maximum propulsion velocity was not significantly affected, as compared to treatment with phentolamine alone (Figure 9.3a).

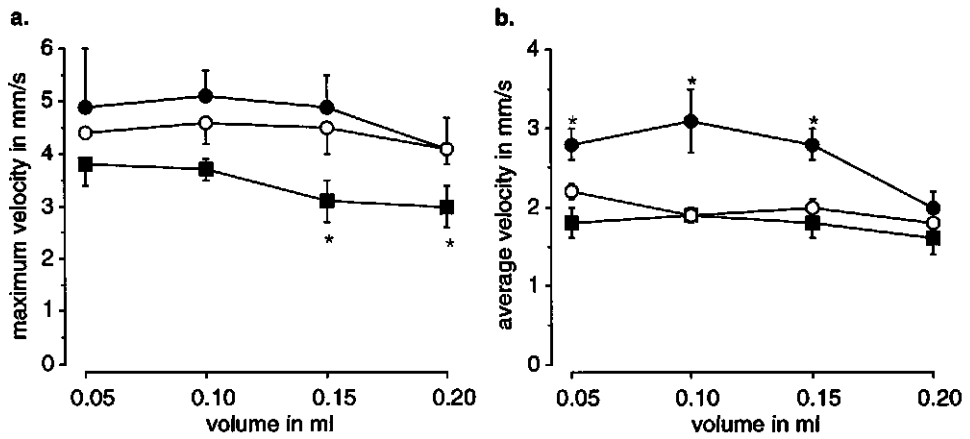


Figure 9.3 Graphical representation of the mean propulsion velocity at different inflation volumes of the balloon. In Figure 9.3a, the maximum propulsion velocity is shown, while in Figure 9.3b the average velocity is depicted. Treatment with the opioid receptor antagonist naloxone (1 mg/kg i.v.) (■) in addition to the α-adrenoceptor antagonist phentolamine (2 mg/kg i.v.) significantly decreased the maximum, but not the average, propulsion velocity as compared to treatment with phentolamine alone (○) (denoted by an asterisk, $P < 0.05$). Treatment with the NO synthase inhibitor N^G-nitro-L-arginine (25 mg/kg i.v.) (●) in addition to phentolamine, however, significantly increased average, but not maximum propulsion velocity as compared to treatment with phentolamine alone. Data points with error bars represent mean ± standard error of the mean.

Discussion

After injection of phentolamine, it was not possible to detect a difference at both maximal and average propulsion velocities of the balloon at the volumes that were studied. The diameter of the balloon ranged from about 4 (0.05 ml) to 8 (0.2 ml) mm, while natural pellets have a diameter of about 5 mm in animals of about 800 g body weight. Hence, varying the balloon diameter around its physiological diameter does not greatly affect propulsion velocity. In isolated segments of the guinea-pig distal colon, it has been shown that the balloon diameter does influence the velocity of propulsion (Frigo & Lecchini, 1970). We have no explanation for these differences. However, it must be stated that the maximum velocity of propulsion in the isolated colon is about 1.5-1.9 mm/s (Frigo & Lecchini, 1970; Costa & Furness, 1976; Krantis & Kerr, 1981), whereas in our model it was about 2-3 times higher. Furthermore, in isolated segments peristalsis occurred spontaneously, whereas *in vivo* α-adrenoceptor blockade was necessary. This exemplifies the differences between *in vitro* and *in vivo*

measurements. In their paper, Frigo & Lecchini (1970) report that the threshold volume of the balloon was about 0.05 ml. However, in some cases they observed propulsion of the empty balloon, as we did in our model.

Almost a century ago, Bayliss & Starling (1899) demonstrated that spontaneous and stimulated movements of the gastrointestinal tract could be inhibited by opening of the peritoneal cavity and by manipulation of the intestines. Abdominal surgery and anaesthesia are known stress factors that induce a temporal intestinal paralysis in animal and man, also named postoperative ileus, which is probably mainly caused by increased sympathetic activity (Dubois, 1988; Livingston & Passaro, 1990). Circulating catecholamines from the adrenals and noradrenaline from the extrinsic sympathetic nerves may stimulate α-adrenoceptors on the enteric nerves. Stimulation of prejunctional α_2-adrenoceptors has been shown to inhibit the release of acetylcholine from enteric neurons (Kojima *et al.*, 1988; Galligan, 1993). The myogenic spontaneous movements are known to be promoted by cholinergic neural activity, i.e. acetylcholine (Maggi & Meli, 1984; Maggi *et al.*, 1985). After blocking the α-adrenoceptors with phentolamine, an increase in tone and in the amplitude of spontaneous phasic movements of the longitudinal coat were observed, and it was possible to evoke peristalsis. In the conscious guinea-pig, however, injection with phentolamine does not alter intestinal motility as assessed by myoelectric activity measurement (Galligan *et al.*, 1986). Hence these observations are in concordance with a tonic sympathetic inhibition of the intestine due to the surgical procedure and the anaesthesia. α-Adrenergic blockade or other sympathicolytic procedures have been used successfully to prevent or improve postoperative ileus in dogs, rats and man (see: Dubois, 1988; Livingston & Passaro, 1990).

Giuliani *et al.* (1993) reported that, in the urethane anaesthetized guinea-pig, after chemical sympathectomy with guanethidine, treatment with the non-selective opioid receptor antagonist naloxone resulted in more reproducible effects during the measurement of circular muscle activity in the distal colon. In the Trendelenburg preparation of the guinea-pig ileum (Trendelenburg, 1917), Van Nueten *et al.* (1976) have shown that naloxone reversed and prevented "fatigue" phenomena that normally occur during peristalsis in this set-up. Endogenous opioid peptides play a neurotransmitter or neuromodulator role in the enteric nervous system (see: Kromer, 1988) and, like exogenous opioids, may inhibit the release of excitatory of inhibitory neurotransmitters (Tonini *et al.*, 1985; Marino *et al.*, 1992; Galligan, 1993). Therefore, we examined the effects of naloxone in our model. Injection of naloxone, after phentolamine, seemed to

slow or hamper propulsion of the balloon, which could be caused by the high tone of the circular muscle after naloxone treatment. Apparently, under our experimental conditions, opioid receptor blockade by naloxone disrupts the balance between inhibitory and excitatory influences of the circular muscle in favour of the excitatory input.

The main inhibitory neurotransmitter of the intrinsic non-adrenergic neurons of the gut is currently thought to be NO, which is formed from L-arginine by Ca^{2+}-activated NO synthase (see: Moncada *et al.*, 1991). NO synthase can be inhibited with N^G-nitro-L-arginine (L-NNA) (Moncada *et al.*, 1991). NO synthase immunoreactive neurons have a widespread distribution the enteric nervous system (Bredt *et al.*, 1990; McConalogue & Furness, 1993), and NO-mediated responses have been extensively studied in many species, amongst which also the guinea-pig (Briejer *et al.*, 1992; Meulemans *et al.*, 1993). In our model, inhibition of the nitrergic transmission by L-NNA in combination with phentolamine induced an increase in average propulsion velocity as compared to treatment with phentolamine alone. Tonini (1993) also observed an increase in propulsion velocity of an artificial pellet in the rabbit isolated colon. Suzuki *et al.* (1994) observed in the isolated guinea-pig ileum, that peristalsis was facilitated by treatment with L-NNA, and they suggested that this was caused by an increase in contractile activity of the circular muscle layer, which is normally inhibited by NO. A similar mechanism could also account for the increase in average propulsion velocity in our model after inhibition of NO synthesis.

The model presented in this paper is the first to allow the evaluation of the effects of drugs on propulsion velocity *in vivo*. Drugs may induce propulsion (phentolamine), or may alter velocity of propulsion in preparations already treated with phentolamine. This model thus adds an important new tool to investigate pharmacological modulation of the motility of the gut.

References

Bayliss WM, Starling EH (1899) The movements and innervation of the small intestine. *J Physiol* 24: 99-143.

Bredt DS, Hwang PM, Snyder SH (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 347: 768-770.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1992) Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*. *Br J Pharmacol* 107: 756-761.

Costa M, Furness JB (1976) The peristaltic reflex: an analysis of the nerve pathways and their pharmacology. *Naunyn-Schmiedeberg's Arch Pharmacol* 294: 47-60.

Dubois A (1988) Small and large intestine: mechanical gastrointestinal obstruction and paralytic ileus. In: *An illustrated guide to gastrointestinal motility* (Kumar D, Gustavsson S Eds.), John Wiley & Sons Ltd., Chichester, pp. 365-382.

Frigo GM, Lecchini S (1970) An improved method for studying the peristaltic reflex in the isolated colon. *Br J Pharmacol* 39: 346-356.

Galligan JJ (1993) Differential inhibition of cholinergic and noncholinergic neurogenic contractions by mu opioid and alpha-2 adrenergic agonists in guinea pig ileum. *J Pharmacol Exp Ther* 264: 375-383.

Galligan JJ, Furness JB, Costa M (1986) Effects of cholinergic blockade, adrenergic blockade and sympathetic denervation on gastrointestinal myoelectric activity in guinea pig. *J Pharmacol Exp Ther* 238: 1114-1125.

Gershon MD, Erde SM (1984) The enteric nervous system of the gut. *Gastroenterology* 80: 1571-1594.

Giuliani S, Lecci A, Giachetti A, Maggi CA (1993) Tachykinins and reflexly evoked atropine-resistant motility in the guinea pig colon *in vivo*. *J Pharmacol Exp Ther* 265: 1224-1231.

Krantis A, Kerr DIB (1981) The effect of GABA antagonism on propulsive activity of the guinea-pig large intestine. *Eur J Pharmacol* 76: 111-114.

Kojima S, Sakato M, Shimo Y (1988) An α_2-adrenoceptor-mediated inhibition of non-adrenergic non-cholinergic inhibitory responses of the isolated proximal colon of the guinea-pig, *Asia Pacific J Pharmacol* 3: 69-75.

Kromer W (1988) Endogenous and exogenous opioids in the control of gastrointestinal motility and secretion. *Pharmacol Rev* 40: 121-162.

Livingston EH, Passaro EP (1990) Postoperative ileus. *Dig Dis Sci* 35: 121-132.

Maggi CA, Meli A (1984) Eserine-induced hypertone of guinea-pig distal colon *in vivo*: a new pharmacological procedure for testing smooth muscle relaxant. *J Pharmacol Meth* 12: 91-96.

Maggi CA, Santicioli P, Manzini S, Meli A (1985) Contribution of neurogenic and myogenic factors to the contractile activity of the guinea-pig distal colon *in vivo* and *in vitro*. *J Auton Pharmacol* 5: 177-187.

Marino F, Creta F, De Ponti F, Giaroni C, Lecchini S, Frigo GM (1993) Opioid pathways exert a tonic restraint in the guinea-pig isolated colon: changes after chronic sympathetic denervation. *J Pharm Pharmacol* 45: 668-670.

McConalogue K, Furness JB (1993) Projections of nitric oxide synthesizing neurons in the guinea-pig colon. *Cell Tissue Res* 271: 545-553.

Meulemans AL, Helsen LF, Schuurkes JAJ (1993) The role of nitric oxide (NO) in 5-HT-induced relaxations of the guinea-pig stomach. *Naunyn-Schmiedeberg's Arch Pharmacol* 348: 424-430.

Moncada S, Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109-142.

Suzuki N, Mizuno K, Gomi Y (1994) Role of nitric oxide in the peristalsis in the isolated guinea-pig ileum. *Eur J Pharmacol* 251: 221-227.

Tonini M, Onori L, Perucca E, Manzo L, De Ponti F, Crema A (1985) Depression by morphine of the excitability of intrinsic inhibitory neurons in the guinea-pig colon. *Eur J Pharmacol* 115: 317-320.

Tonini M (1993) Nitric oxide dependent and independent mechanisms underlie separate neurogenic inhibitory functions in the rabbit large intestine. *Proc Aust Neurosci Soc* 4: 79P.

Trendelenburg P (1917) Physiologische und pharmakologische Versuche über die Dünndarmperistaltik. *Naunyn-Schmiedeberg's Arch Exp Pathol Pharmakol* 81: 55-129.

Van Nueten JM, Janssen PAJ, Fontaine J (1976) Unexpected reversal effects of naloxone on the guinea pig ileum. *Life Sci* 18: 803-810.

General discussion

Although the gastrointestinal motility-stimulating action of the prokinetic substituted benzamides is already known for more than 30 years, it is still not completely understood how they evoke this effect. A large part of this gap in our understanding is due to the fact that gastrointestinal motility (disorders) itself is still far from completely understood. Another part is caused by the limited attention the field of 5-HT receptor research received in the 1970s. Only in the second part of the 1980s, 5-HT and its receptors regained attention from the scientific community, which soon culminated in an avalanche of papers in the field. Selective tools for 5-HT receptors, but also for other neurotransmitter receptors, have become available in recent years. Against this background, the investigations as described in this thesis were done.

The occurence of 5-HT receptor subtypes in the guinea-pig proximal colon was found to be very similar to the situation in the ileum (see General introduction), which is the classical model for *in vitro* studies. To date, no important role has been implicated for the 5-HT_{2A} receptors which are present on the smooth muscle of both the ileum and the colon (see Figure II). Like in the ileum, also in the colon 5-HT_3 and 5-HT_4 receptors are present on the enteric nerves. Both receptor subtypes were shown to be involved in the contraction to 5-HT of the colon. In the ileum, 5-HT_3 receptors mediate by far the larger part of the contraction to 5-HT, while in the colon the relative contribution of 5-HT_3 and 5-HT_4 receptors seems equal. These differences could reflect different receptor distributions. In the colon, the contraction to 5-HT was highly sensitive to atropine, suggesting that neuronally released acetylcholine mediates the contraction, but involvement of substance P could not be excluded. As substance P was found to evoke a contraction via NK_1 receptors, future studies could be designed to investigate the relative contribution of this tachykinin in 5-HT_3 and 5-HT_4 receptor-mediated contraction. Both cisapride and R 76 186 were found to cause a contraction of the colon through stimulation of 5-HT_4 receptors. Thus, with novel selective NK_1 receptor antagonists like CP 96-345, future studies could assess whether non-cholinergic transmission is facilitated next to cholinergic transmission. As pointed out in Chapter 1, the contribution of the non-cholinergic neurotransmitters might turn out to be important for the understanding of the mechanism of action of the substituted benzamides.

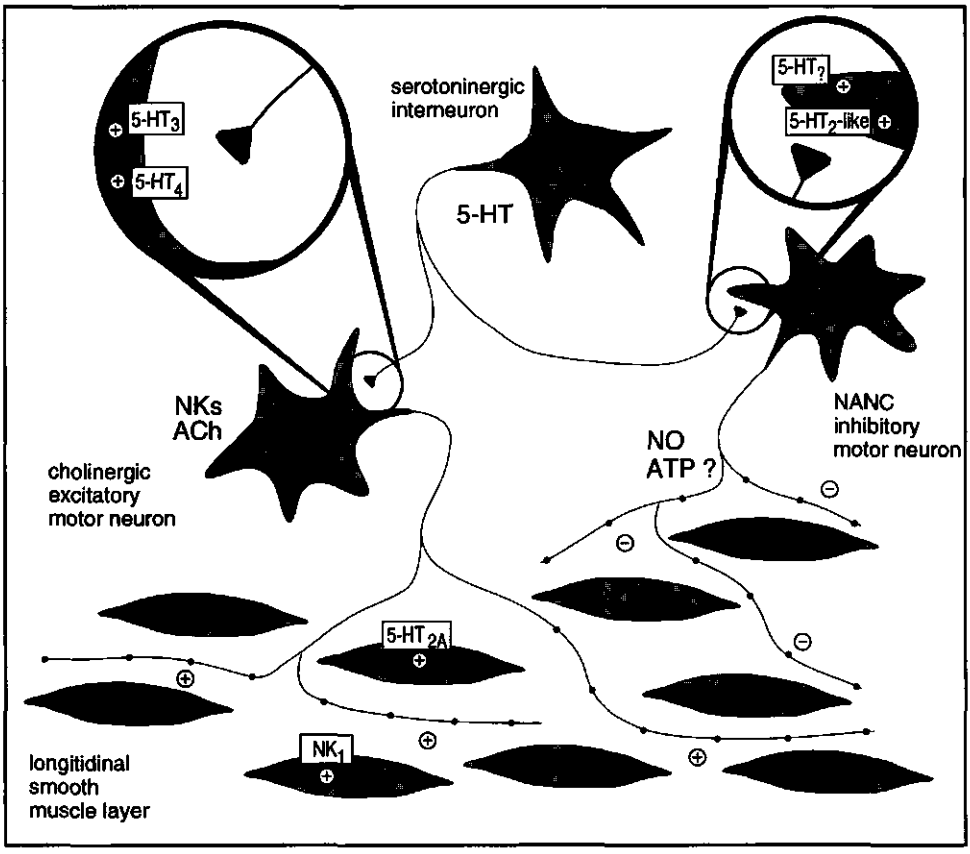


Figure II Schematic representation of the innervation of the guinea-pig proximal colon, as deduced from the experiments described in this thesis. The longitudinal muscle layer and some myenteric neurons are depicted. The scheme has been greatly simplified as only the excitatory serotonergic, cholinergic and inhibitory non-adrenergic non-cholinergic (NANC) neurons are shown. Serotonin (5-HT) released from interneurons and consecutively released neurotransmitters can stimulate a variety of both neuronal and smooth muscle receptors in the colon causing either stimulation (+) or inhibition (-). After stimulation of 5-HT_3 and 5-HT_4 receptors, the release of acetylcholine (ACh) and neurokinins (NKs) is enhanced. 5-HT can also stimulate 5-HT_2-like and unknown 5-HT receptors on the NANC inhibitory neurons inducing the release of the neurotransmitters nitric oxide (NO) and possibly adenosine triphosphate (ATP) or related purines. On the smooth muscle, it was shown that 5-HT_{2A} and neurokinin NK_1 receptors are present, which mediate contraction.

In contrast to the ileum, 5-HT also induced relaxation by stimulation of a novel high affinity 5-HT_2-like receptor and a low affinity 5-HT receptor on the nerves (see Figure II). Since the beginning of the 1970s, many researchers have attempted to establish the non-adrenergic non-cholinergic inhibitory neurotransmitter in the gastrointestinal tract. It was shown that stimulation of the

5-HT_2-like receptors evokes the release of nitric oxide. The low affinity 5-HT receptor, which could not be characterized, probably accounts for the release of adenosine triphosphate (ATP) (see Figure II). The involvement of a peptide like vasoactive intestinal polypeptide is not likely. It was found that some benzamides, e.g. cisapride, cinitapride, clebopride and dazopride, selectively enhance the ATP-mediated part of the relaxation to 5-HT. Furthermore, it was suggested that they were an antagonist at the 5-HT_2-like receptor, which might mask enhancing effects on NO release. Although facilitation of relaxation next to the established facilitation of cholinergic contraction could theoretically contribute to the prokinetic effects of the benzamides (see Figure III), such an involvement has yet to be demonstrated in relevant *in vivo* models.

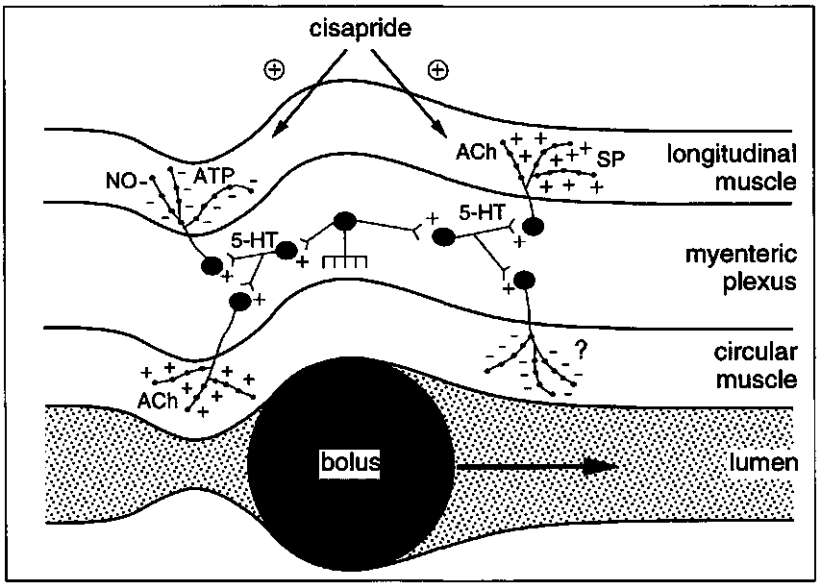


Figure III Schematical simplified representation of the hypothesis that substituted benzamides like cisapride exert their prokinetic action through facilitation of both excitatory (acetylcholine (ACh), substance P (SP)) and inhibitory (adenosine triphosphate (ATP) and possibly nitric oxide (NO)) neurotransmission upon demand. The demand is set by sensory neurons which are sensitive to stretch or pressure exerted by the intraluminal bolus. These sensory neurons consecutively release transmitters to excite serotonergic (5-HT) interneurons, which in turn activate inhibitory or excitatory neurons. Such a circuit must be repeated many times along the intestine to ensure ongoing peristalsis. The neurotransmitter(s) mediating relaxation of the circular smooth muscle coat have not been established yet.

R 76 186 appeared to be a specific 5-HT_4 receptor agonist, but cisapride evoked part of its contraction on the colon *via* a direct action on the smooth muscle. Although we attempted to characterize this part of the response, it is still not known how this effect is caused, and whether this effect contributes to its prokinetic actions *in vivo*. The interaction of benzamides with the 5-HT_4 receptors is a complicated one, but again the relevance of this finding has yet to be established.

The *in vivo* model of faecal pellet propulsion in the guinea-pig distal colon as presented in this thesis could prove a valuable model to test the above described hypotheses. Comparison of the effect of the specific benzamide R 76 186, cisapride and non-benzamide 5-HT_4 receptor agonists might teach us more on the mechanism of action of substituted benzamides.

Conclusions

The conclusions to the aims of the investigations as described in this thesis (see: General introduction), are thus:

- *in the isolated guinea-pig colon (longitudinal muscle):*
  1. 5-HT induces both contraction *via* 5-HT_{2A} receptors on the smooth muscle and 5-HT_3 and 5-HT_4 receptors on the enteric nerves, and relaxation *via* unidentified 5-HT receptors on the nerves;
  2. NO and ATP, but not VIP, are involved in the relaxation to 5-HT after neuronal 5-HT receptor stimulation; acetylcholine and possibly a non-cholinergic substance mediate the neurogenic contraction to 5-HT;
  3. substance P induces a contraction *via* tachykinin NK_1 receptors on the smooth muscle;
  4. cisapride and the structural analog, R 76 186 induce contractions through neuronal 5-HT_4 receptor stimulation; however, cisapride also has a direct effect on the smooth muscle which remains unidentified;
  5. two neuronal 5-HT receptors seem to be involved in the relaxation to 5-HT; the low affinity 5-HT receptor remains unidentified, but the high affinity 5-HT receptor is proposed to be a novel 5-HT_2-like receptor;
  6. cisapride and some other benzamides selectively enhanced the ATP-mediated part of the 5-HT-induced relaxations, while they inhibited the NO-mediated part.

- *in the guinea-pig colon in vivo:*
  7. a model in the anaesthetized guinea-pig for the study of peristalsis in the distal colon was established, which could prove valuable for the assessment of drug effects which interfere with gastrointestinal motility.

Summary

In vitro pharmacological studies of gastrointestinal tissues have classically been done on guinea-pig ileum. It is known however that the guinea-pig colon has different properties. For example, in the colon non-adrenergic non-cholinergic neurotransmitters seem to play a bigger role as compared to the ileum, and the colon develops a spontaneous tone, in contrast to the ileum. The gastrointestinal motility stimulating benzamides are known to interfere with several 5-hydroxytryptamine (5-HT) receptor subtypes either as agonist or antagonist. Agonism at neuronal 5-HT_4 receptors has been proposed as their common property to explain their prokinetic effects, thus facilitating cholinergic transmission. However, many questions as to their mechanism of action still remain. The bulk of the studies in which this issue was investigated used the guinea-pig ileum. The studies in this thesis describe an investigation into the effects of prokinetic benzamides and 5-HT and their interaction on the guinea-pig colon.

Chapter 1 describes and evaluates in detail the studies and the subsequent theories on the mechanism of action of the substituted benzamides that have been proposed during the last 30 years. In the 1970s, it was proposed that the prokinetic benzamide metoclopramide, and later clebopride act as antagonists of peripheral dopamine D_2 receptors, but in the early 1980s this theory was abandoned. The benzamides that emerged in this decade (cisapride, renzapride) had a much lower affinity for dopamine D_2 receptors, but a higher potency to stimulate motility than metoclopramide. Moreover, they still had a facilitory action on cholinergic neurotransmission in the guinea-pig ileum. In the late 1980s a fourth 5-HT receptor subtype was discovered, named 5-HT_4. It was found that the benzamides are agonists at this 5-HT_4 receptor, and that stimulation of 5-HT_4 receptors on the myenteric neurons of the guinea-pig ileum causes facilitation of cholinergic transmission. However, though the benzamides are effective motility stimulants in man and animal, neuronal 5-HT_4 receptors in the rat and human gastrointestinal tract have not yet been established. Also other questions as to their mechanism of action are dealt with in this chapter.

Chapter 2 describes a study in which it was investigated which 5-HT receptors mediate the responses to 5-HT in the longitudinal muscle layer of the guinea-pig proximal colon, using selective 5-HT receptor antagonists and 5-HT analogues. 5-HT as well as its analogues induced both contractions and relaxations. Tetrodotoxin (TTX) abolished the relaxations, and it inhibited the contractions to all agonists. In the presence of TTX, blockade of either 5-HT_{2A} or 5-HT_3 receptors reduced the contractions to 5-HT, whereas blockade of both 5-HT receptor types at

the same time abolished them. In the absence of TTX, the contractions to 5-HT were inhibited by antagonists at 5-HT_{2A}, 5-HT_3 and also 5-HT_4 receptors. Contractions to α-methyl-5-HT did not seem to be sensitive to 5-HT_{2A} receptor blockade, but in the presence of TTX the contractions were abolished by the 5-HT_{2A} receptor antagonist. A 5-HT_3 receptor antagonist abolished contractions to 2-methyl-5-HT. In the presence of TTX, a 5-HT_{2A} receptor antagonist abolished contractions to 5-methoxytryptamine (5-MeOT), and in the absence of TTX the contractions to 5-MeOT were highly sensitive to blockade of 5-HT_4 receptors. Blockade of either 5-HT_1-like, 5-HT_{2A}, 5-HT_3 or 5-HT_4 receptors did not abolish the relaxations to 5-HT or 5-MeOT.

It was concluded that 5-HT induces contractions of the longitudinal muscle of the guinea-pig proximal colon, through the stimulation of 5-HT_{2A} receptors on the smooth muscle and 5-HT_3 receptors and neuronal 5-HT_4 receptors. 5-HT evokes relaxations via an unknown neuronal receptor.

To establish the receptor that mediates contraction to substance P, the effects of three tachykinin NK_1 receptor antagonists and a NK_2 receptor antagonist against substance P-induced contractions of the guinea-pig proximal colon longitudinal muscle were investigated (**Chapter 3**). Atropine, TTX and phosphoramidon did not affect the concentration-response curve for substance P (pEC_{50} = 7.8). The NK_1 receptor antagonist, 2S,3S-cis-CP 96345, competitively inhibited the contractions due to substance P (pA_2 = 8.5; pK_b = 8.9), but at higher concentrations (≥ 0.3 μM), CP 96345 also depressed the concentration-response curve for methacholine. The species-selective NK_1 receptor antagonists, WIN 51708 and WIN 62577, and the NK_2 receptor antagonist, SR 48968, had no effect.

It was concluded that substance P induces contractions through the stimulation of NK_1 but not NK_2 receptors on the smooth muscle cells. In future studies, NK_1 receptor antagonists could thus be used to study the involvement of substance P in benzamide-induced responses in this preparation.

The purpose of the study described in **Chapter 4** was to investigate whether the effects of cisapride and its close structural analogue R 76 186 on the isolated guinea-pig proximal colon are mediated through 5-HT_4 receptors. Both cisapride and R 76 186 induced contractions which yielded monophasic concentration-response curves (cisapride: EC_{50} = 0.11 μM; R 76 186: EC_{50} = 24 nM). Blockade of either 5-HT_2 or 5-HT_3 receptors did not affect the responses to cisapride, but 5-HT_4 receptor antagonists depressed the concentration-response curve to cisapride (to about 50 %), and the curve to R 76 186 was shifted to the right. The

estimated pA_2 values were 6.6 (tropisetron), 6.3 (DAU 6285), and 7.5 (SDZ 205-557). However, none of these antagonisms was purely competitive as higher concentrations of these antagonists depressed the curve to R 76 186. Desensitization of the 5-HT_4 receptor with 5-MeOT inhibited the responses to cisapride, and abolished those to R 76 186. The contractions to cisapride and R 76 186 were sensitive to mutual antagonism, depressing their concentration-response curves.

It was concluded that both cisapride and R 76 186 are agonists at the 5-HT_4 receptor, though cisapride also uses a non-5-HT mechanism. R 76 186 is a selective and potent 5-HT_4 receptor agonist.

In **Chapter 5** it was investigated whether nitric oxide (NO) is involved in the neurogenic relaxations to 5-HT on the guinea-pig colon. Antagonists to block the contractile responses to 5-HT *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors were present throughout the experiments and methacholine was administered to elevate tone. Under these conditions, 5-HT concentration-dependently induced relaxations (EC_{50} = 258 nM). The relaxations were inhibited by metergoline and methiothepine and abolished by TTX. Guanethidine did not affect them. N^G-nitro-L-arginine (L-NNA) inhibited the responses to 5-HT (IC_{50} = 18.7 μM); at the highest 5-HT concentration a maximum inhibition of about 75 % was observed with 0.3 mM L-NNA. This inhibition was reversed with L-arginine. Relaxations to glyceryl trinitrate (GTN) were not inhibited by L-NNA. Haemoglobin inhibited the relaxations to 5-HT and GTN, but not those to isoprenaline (ISO). Methylene blue inhibited the relaxations to 5-HT but did not affect those caused by GTN or ISO.

It was concluded that 5-HT induces relaxations that involve NO. We also confirmed that 5-HT induces these relaxations via (a) 5-HT_1 receptor subtype(s), located on neurons.

Subsequently, in **Chapter 6** it is described whether the inhibitory neurotransmitters adenosine 5'-triphosphate (ATP) and/or vasoactive intestinal polypeptide (VIP) could be involved as well in the neurogenic relaxation to 5-HT. Under the same conditions as described above, ATP, VIP and 5-HT induced concentration-dependent relaxations. TTX, L-NNA and the combination treatment did not inhibit the relaxations induced by VIP or 0.3-3 μM ATP but reduced those by 10 μM ATP. Suramin strongly inhibited the relaxations to ATP and VIP. L-NNA and suramin also inhibited the relaxations to 5-HT. In the presence of L-NNA, suramin did not significantly inhibit the relaxations to 5-HT. Suramin did not

affect the relaxations to ISO, GTN or exogenous NO, demonstrating its specificity. Apamin inhibited both the relaxations to ATP (by 70-100 %) and to 5-HT; relaxations to ISO were partially inhibited, indicating a non-specific component in the inhibitory action of apamin. However, relaxations to exogenous VIP, NO and to GTN were not inhibited. In the presence of L-NNA, apamin inhibited the relaxations to 5-HT only at 30 μM. α,β-methylene-ATP (α,β-Me-ATP) did not desensitize the responses to ATP. Reactive blue 2 affected the relaxations to ISO at concentrations necessary to significantly inhibit the relaxations to ATP. Thus, it was not possible to test either α,β-Me-ATP or reactive blue 2 against the relaxations to 5-HT. α-Chymotrypsin and trypsin almost abolished the relaxations to VIP, but did not affect those to ISO and 5-HT. The VIP receptor antagonists [p-Cl-D-Phe^{6},Leu^{17}]VIP and VIP_{10-28} did not affect the concentration-response curve to VIP and were hence not tested against 5-HT. Phosphoramidon had no effect on the relaxations to VIP or 5-HT.

It was concluded that on the guinea-pig colon longitudinal muscle, VIP and ATP induce relaxation via a direct effect of the smooth muscle, not involving NO. 5-HT-induced relaxations are mediated by NO as well as by a substance which is sensitive to inhibition by suramin and apamin. It is suggested that this substance is ATP and not a peptide like VIP.

Again under the same conditions as described for chapter 5 and 6, **Chapter 7** describes the characterization of the receptor(s) that mediate the neurogenic relaxation to 5-HT on the guinea-pig colon. The relaxations to 5-HT yielded a biphasic concentration-response curve. Other tryptamines were also agonists with the following rank order of potency: 5-HT > 5-carboxamidotryptamine = 5-MeOT ≥ α-methyl-5-HT (partial agonist) > tryptamine (partial agonist). 5-Hydroxytryptophan, 2-methyl-5-HT and N-methyl-tryptamine were virtually inactive as agonists. The concentration-response curve to 5-HT was not affected by pargyline, citalopram, phentolamine, a tenfold increase of the ketanserin concentration, or by the 5-HT_4 receptor antagonists SDZ 205-557 and SB 204070. 8-OH-DPAT, RU 24969, WB 4101, mCPP, TFMPP, flesinoxan, sumatriptan and MK212 were inactive as 5-HT receptor agonists; some were found to antagonize methacholine-induced contractions. The first phase of the concentration-relaxation curve to 5-HT was inhibited by the following drugs: metergoline (pA_2 = 8.8, against 5-MeOT 9.3), methysergide (non-surmountable; NS), methiothepin (NS), spiroxatrine (NS), MK212 (NS), mesulergine (7.8), mCPP (7.1), mianserin (7.0), ritanserin (8.9), rauwolscine (7.0), yohimbine (6.2), 1-NAP (7.7) and RU 24969 (6.4), but not by NAN-190, spiperone, sumatriptan, 8-OH-DPAT and flesinoxan.

When compared with pharmacological affinity and efficacy profiles at the currently recognized 5-HT receptor subtypes, the guinea-pig colon high affinity 5-HT receptor correlated only with receptors of the 5-HT_2 group (significantly with 5-HT_{2C} and near-significantly with 5-HT_{2A}). The receptor under study however also displayed pronounced pharmacological differences with these receptors.

It was therefore concluded that two 5-HT receptors are involved in the neurogenic relaxation to 5-HT: the high affinity receptor could be considered a 5-HT_2-like receptor, but the low affinity 5-HT receptor remains unidentified.

In **Chapter 8**, the effects of cisapride and structural analogues on the 5-HT-induced relaxations were investigated under conditions as described above (Chapters 5, 6 and 7). Cisapride (0.1-1 μM) enhanced the second phase of the concentration-response curve to 5-HT by about 20-40 %, while, from 0.3 μM onwards it inhibited the first phase. Also in the presence of cisapride, TTX abolished the relaxations to 5-HT. Cisapride did not affect the concentration-response curves to ISO, GTN, nitroprusside or exogenous ATP, demonstrating its specificity. The 5-HT-relaxation enhancing effects of cisapride were not mimicked by phentolamine, NAN-190, spiperone, citalopram, paroxetine, pargyline or SDZ 205-557. In the presence of L-NNA, cisapride still enhanced the remaining relaxations to 5-HT (2-3 fold). However, in the presence of suramin, cisapride could not enhance the relaxations to 5-HT. In the presence of L-NNA, the cisapride-enhanced relaxations to 5-HT could be inhibited by about 90 % by suramin.

It was concluded that cisapride selectively facilitates the suramin-sensitive, ATP-mediated, part of the relaxation to 5-HT via an unidentified effect on intramural nerves.

A novel method for the study of colonic peristalsis in the anaesthetized guinea-pig was developed (**Chapter 9**). After laparotomy, a rubber balloon was inserted into the lumen of the distal colon and inflated with 0.05-0.2 ml of water to mimic faecal pellet size. Thus, movement of the balloon and change in length of the longitudinal muscle could be assessed by isotonic recording. Inflation of the balloon *per se* did not induce propulsion of the balloon. Treatment with phentolamine (2 mg/kg i.v.) induced peristalsis and balloon displacement. This suggests that sympathetic nerve hyperactivity, probably due to anaesthesia and surgery, exerts a tonic inhibitory influence on the colon, which prevents co-ordinated motor activity. Naloxone (1 mg/kg i.v.) did not promote peristalsis

when given alone. However, when naloxone was administered in addition to phentolamine, a decrease in the maximum, but not the average velocity of propulsion was observed compared to treatment with phentolamine alone. Treatment with L-NNA (25 mg/kg i.v.) in addition to phentolamine increased the average, but not the maximum, pellet propulsion velocity as compared to treatment with phentolamine alone. Irrespective of the treatment schedule employed, the velocity of propulsion was not dependent upon the inflation volume of the balloon.

This model provides the first method to evaluate the effects of benzamides and 5-HT receptor (ant)agonists as described in the previous chapters, on colonic propulsion in the guinea-pig *in vivo* .

Samenvatting

Farmacologische *in-vitro*-studies werden in het verleden doorgaans uitgevoerd met voornamelijk geïsoleerde stukjes ileum van de cavia. Het is echter bekend dat het colon van de cavia andere eigenschappen heeft dan het ileum. Zo lijken bijvoorbeeld de niet-adrenerge niet-cholinerge neurotransmitters een grotere rol te spelen in het colon vergeleken met het ileum, en ontwikkeld het colon een spontane tonus, in tegenstelling tot het ileum. De benzamiden, die de gastrointestinale motiliteit stimuleren, gaan een interactie aan met verscheidene subtypen receptoren voor 5-hydroxytryptamine (5-HT), als agonist of als antagonist. Stimulatie van neuronale 5-HT_4-receptoren gevolgd door facilitatie van cholinerge neurotransmissie is het mechanisme dat voorgesteld is verantwoordelijk te zijn voor de prokinetische effecten van de benzamiden. Er zijn echter nog veel vragen omtrent het werkingsmechanisme van de benzamiden. De overgrote meerderheid van de studies waarin deze kwestie werd bestudeerd maakten gebruik van het ileum van de cavia. De studies die in dit proefschrift aan de orde komen beschrijven onderzoek naar de effecten van prokinetische benzamiden en 5-HT en hun interactie op het colon van de cavia.

Chapter 1 beschrijft en evalueert gedetailleerd de studies en de theorieën die de laatste 30 jaar opgang hebben gedaan betreffende het werkingsmechanisme van de gesubstitueerde benzamiden. In de jaren '70 werd voorgesteld dat het prokinetische benzamide metoclopramide, en later ook clebopride, werkt als antagonist op perifere dopamine-D_2-receptoren, maar in het begin van de tachtiger jaren was deze theorie alweer verlaten. De benzamiden die in dit decade ontwikkeld werden (cisapride, renzapride) hadden een veel lagere affiniteit voor dopamine-D_2-receptoren, maar waren tevens potenter in hun prokinetische werking. De faciliterende werking op cholinerge neurotransmissie in het ileum van de cavia was echter behouden gebleven. Aan het einde van de tachtiger jaren werd er een nieuw type 5-HT-receptor ontdekt, die 5-HT_4 gedoopt werd. Het bleek dat de benzamiden agonisten zijn op deze receptor, en dat stimulatie van deze receptoren op het ileum van de cavia facilitatie van cholinerge transmissie veroorzaakt. Hoewel gebleken is dat zowel in de mens als het dier de benzamiden effectieve stimulantia van de gastro-intestinale motoriek zijn, zijn neuronale 5-HT_4-receptoren in de tractus digestivus van de rat en de mens (nog) niet gevonden. Ook vele andere vraagtekens die nog altijd rondom het werkingsmechanisme bestaan komen in dit hoofdstuk aan de orde.

Chapter 2 beschrijft een studie naar de typen 5-HT-receptoren die de effecten van 5-HT op de longitudinale spierlaag van het proximale colon van de cavia

mediëren. Hiertoe werden analogen en selectieve antagonisten van 5-HT-receptoren gebruikt. Zowel 5-HT als zijn analogen induceerde contracties en relaxaties. Tetrodotoxine (TTX) blokkeerde de relaxaties en remde de contracties, die geïnduceerd werden door alle geteste agonisten. Blokkade van 5-HT_{2A}- of 5-HT_3-receptoren in de aanwezigheid van TTX remde de 5-HT-geïnduceerde contracties, terwijl blokkade van beide receptortypen te zelfder tijd een volledige remming gaf. In afwezigheid van TTX werden de contracties die door 5-HT geïnduceerd werden, geremd door antagonisten van 5-HT_{2A}-, 5-HT_3- en 5-HT_4-receptoren. Contracties na toediening van α-methyl-5-HT bleken niet gevoelig te zijn voor blokkade van de 5-HT_{2A}-receptor maar in aanwezigheid van TTX werden de contracties volledig geremd door de 5-HT_{2A}-receptor-antagonist. Een 5-HT_3-receptor-antagonist remde de contracties geïnduceerd door 2-methyl-5-HT. In aanwezigheid van TTX blokkeerde een 5-HT_{2A}-receptor-antagonist de contracties na toediening van 5-methoxytryptamine (5-MeOT), en in afwezigheid van TTX werden de contracties geremd door een 5-HT_4-receptor-antagonist. Blokkade van 5-HT_1-achtige, 5-HT_{2A}-, 5-HT_3- of 5-HT_4-receptoren had geen volledige remming van de relaxaties geïnduceerd door 5-HT of 5-MeOT tot gevolg.

De conlcusie was dat 5-HT contracties van de longitudinale spierlaag van het proximale colon van de cavia induceert via 5-HT_{2A}-receptoren op het gladde spierweefsel, 5-HT_3-receptoren en neuronale 5-HT_4-receptoren. De relaxaties worden gemedieerd via een onbekende neuronale 5-HT-receptor.

De effecten van drie tachykinine NK_1-receptor-antagonisten en een NK_2-receptor-antagonist op substance-P-geïnduceerde contracties van de longitudinale spierlaag van het colon van de cavia werden in deze studie onderzocht (**Chapter 3**). Atropine, TTX en fosforamidon hadden geen effect op de concentratie-responscurve van substance P (pEC_{50} = 7.8). De NK_1-receptor-antagonist 2S,3S-cis-CP 96345 remde competitief de contracties die door substance P geïnduceerd werden (pA_2 = 8.5; pK_b = 8.9). Bij hogere concentraties (≥ 0.3 μM) remde CP 96345 echter ook contracties geïnduceerd door methacholine. De species-selectieve NK_1-receptor-antagonisten WIN 51708 en WIN 62577 en de NK_2-receptor-antagonist SR 48968 hadden geen effect.

Geconcludeerd werd dat substantie P zijn contractiele werking op de longitudinale spierlaag van de cavia medieert via NK_1- en niet via NK_2-receptoren op de gladde-spiercellen in dit preparaat.

Het doel van de studie zoals beschreven in **Chapter 4** was na te gaan of cisapride en zijn structuuranaloog R 76 186 hun effect op het geïsoleerde colon van de

cavia uitoefenen via 5-HT_4-receptoren. Zowel cisapride als R 76 186 induceerde contracties, hetgeen monofasische concentratie-responscurven opleverde (cisapride: EC_{50} = 0.11 µM; R 76186: EC_{50} = 24 nM). Antagonisten van 5-HT_2- en 5-HT_3-receptoren hadden geen effect op de responsen geïnduceerd door cisapride en R 76 186, maar 5-HT_4-receptor-antagonisten veroorzaakten een depressie van de concentratie-responscurve van cisapride (tot ca. 50 %), en de curve van R 76 186 werd naar rechts verschoven. De geschatte pA_2-waarden waren 6.6 (tropisetron), 6.3 (DAU 6285) en 7.5 (SDZ 205-557). Geen van de remmingen was echter zuiver competitief van aard, daar hogere concentraties van deze antagonisten de curve van R 76 186 deprimeerden. Desensitisatie van de 5-HT_4-receptoren met 5-MeOT remde de contracties geïnduceerd door cisapride gedeeltelijk, en remde die geïnduceerd door R 76 186 volledig. De contracties geïnduceerd door R 76 186 en cisapride waren onderhevig aan wederzijds antagonisme, waarbij er een depressie van de curven optrad.

Er werd geconcludeerd dat zowel cisapride als R 76 186 agonisten zijn van de 5-HT_4-receptor, hoewel cisapride ook nog via een mechanisme werkt dat niet met 5-HT-receptoren te maken heeft.

In **Chapter 5** wordt een onderzoek beschreven naar de betrokkeheid van stikstofoxide (NO) bij de neurogene relaxaties, die door 5-HT op het proximale colon worden geïnduceerd. Gedurende alle experimenten waren er antagonisten in de badvloeistof om contracties gemediëerd via 5-HT_{2A}-, 5-HT_3- en 5-HT_4-receptoren te blokkeren, en methacholine werd telkens toegevoegd om de tonus van de strips te verhogen. Onder deze omstandigheden induceerde 5-HT relaxaties (EC_{50} = 258 nM). Deze relaxaties werden geremd door metergoline en methiothepine en geblokkeerd door TTX. Guanethidine had geen effect. N^G-nitro-L-arginine (L-NNA) remde de relaxaties geïnduceerd door 5-HT (IC_{50} = 18.7 µM); bij de hoogste concentratie 5-HT bedroeg de remming door 0.3 mM L-NNA ca. 75 %. De inhibitie kon gedeeltelijk opgeheven worden door tevens L-arginine toe te voegen. Relaxaties geïnduceerd door nitroglycerine (GTN) werden niet geremd door L-NNA. Hemoglobine remde de relaxaties geïnduceerd door 5-HT en GTN, maar niet die door isoprenaline (ISO). Methyleenblauw remde de relaxaties geïnduceerd door 5-HT maar niet die door GTN of ISO.

Geconcludeerd werd dat 5-HT relaxaties induceert via NO. De resultaten bevestigen, dat de neuronale 5-HT-receptor(en) die hierbij betrokken zijn, van het 5-HT_1-achtige subtype zijn.

Vervolgens wordt in **Chapter 6** beschreven of de inhibitoire neurotransmitters adenosine 5'-trifosfaat (ATP) en/of vasoactief intestinaal polypeptide (VIP) betrokken zouden kunnen zijn bij de 5-HT-geïnduceerde neurogene relaxaties. Onder dezelfde omstandigheden als boven beschreven induceerden 5-HT, ATP en VIP concentratie-afhankelijke relaxaties. TTX, L-NNA en de combinatie van beide hadden geen effect op de relaxaties geïnduceerd door VIP of door 0.3-3 μM ATP maar reduceerde de relaxaties geïnduceerd door 10 μM ATP. Suramine remde de ATP- en VIP-geïnduceerde relaxaties in sterke mate. L-NNA en suramine remden ook de relaxaties geïnduceerd door 5-HT. In aanwezigheid van L-NNA remde suramine de relaxaties geïnduceerd door 5-HT niet significant. Suramine had geen effect op de relaxaties na toediening van ISO, GTN of exogeen NO, hetgeen de specificiteit van suramine aantoont. Apamine remde zowel de ATP- (tot 70-100 %) als de 5-HT-geïnduceerde relaxaties. De relaxaties geïnduceerd door ISO werden gedeeltelijk geremd, hetgeen een niet-specifieke component in het remmende effect van apamine aantoont. De relaxaties na toediening van exogeen VIP, NO of GTN werden echter niet geremd. In aanwezigheid van L-NNA remde apamine de 5-HT-geïnduceerde relaxaties alleen bij 30 μM 5-HT. α, β-methyleen-ATP (α, β-Me-ATP) desensitiseerde de respons van ATP niet. Reactive blue 2 remde in concentraties benodigd om ATP-geïnduceerde relaxaties te remmen tevens de relaxaties op ISO-toediening. Het was dus niet zinvol om α, β-Me-ATP of reactive blue 2 te testen op 5-HT-geïnduceerde relaxaties. α-Chymotrypsine en trypsine remden de relaxaties geïnduceerd door VIP bijna volledig, maar hadden geen effect op ISO- en 5-HT-geïnduceerde relaxaties. De VIP-receptor-antagonisten [p-Cl-D-Phe^{6},Leu^{17}]VIP en VIP$_{10-28}$ hadden geen effect op de concentratie-responscurve van VIP en werden daarom niet verder getest op die van 5-HT. Fosforamidon had geen effect op de VIP- en 5-HT-geïnduceerde relaxaties.

Er werd geconcludeerd dat VIP en ATP een relaxatie van het colon van de cavia induceren door een direct effect op de gladde-spiercellen, zonder betrokkenheid van NO. De 5-HT-geïnduceerde relaxaties worden zowel door NO gemedieerd als door een substantie die gevoelig is voor remming door suramine en apamine. Deze substantie is waarschijnlijk ATP en geen peptide zoals VIP.

Eveneens onder dezelfde condities als beschreven in Chapter 5 en 6, wordt in **Chapter 7** de karakterisering van de 5-HT-receptor beschreven die de neurogene relaxatie van 5-HT medieert. De concentratie-responscurve van de 5-HT-geïnduceerde relaxaties was bifasisch. Ondere tryptaminen waren ook agonisten met de volgende rangorde van potentie: 5-HT > 5-carboxamidotryptamine = 5-MeOT

≥ α-methyl-5-HT (partial agonist) > tryptamine (partial agonist). 5-Hydroxytryptophan, 2-methyl-5-HT en N-methyltryptamine waren nagenoeg inactief als agonist. The concentratie-responscurve van 5-HT werd niet veranderd door de aanwezigheid van pargyline, citalopram, fentolamine, een tienvoudige toename van de concentratie ketanserine, of door de 5-HT_4-receptor-antagonisten SDZ 205-557 en SB 204070. 8-OH-DPAT, RU 24969, WB 4101, mCPP, TFMPP, flesinoxan, sumatriptan en MK212 waren inactief als 5-HT-receptor-agonist; sommige van voornoemde stoffen echter remden methacholine-geïnduceerde contracties. De eerste fase van de curve van 5-HT werd geremd door de volgende stoffen: metergoline (pA_2 = 8.8, voor 5-MeOT 9.3), methysergide (depressie: NS), methiothepine (NS), spiroxatrine (NS), MK212 (NS), mesulergine (7.8), mCPP (7.1), mianserine (7.0), ritanserine (8.9), rauwolscine (7.0), yohimbine (6.2), 1-NAP (7.7) en RU 24969 (6.4), maar niet door NAN-190, spiperon, sumatriptan, 8-OH-DPAT en flesinoxan. Bij vergelijking van de farmacologische affiniteits- en efficaciteitsprofielen met de thans bekende subtypen 5-HT-receptoren werd alleen een correlatie met receptoren uit de 5-HT_2-groep gevonden (significant met 5-HT_{2C}, bijna-significant met 5-HT_{2A}). De receptor onder studie vertoonde echter ook duidelijke farmacologische verschillen.

Het werd daarom geconcludeerd dat twee typen 5-HT-receptor de neurogene relaxaties die geïnduceerd worden door 5-HT mediëren: de 5-HT-receptor die een hoge affiniteit heeft voor 5-HT kan als een 5-HT_2-achtige receptor beschouwd worden, maar de andere werd niet geïdentificeerd.

In **Chapter 8** wordt het onderzoek naar de effecten van cisapride en structurele analogen op de 5-HT-geïnduceerde relaxaties beschreven. De omstandigheden waaronder de studie gebeurde waren identiek aan die bij de boven beschreven studies (Chapters 5, 6 en 7). Cisapride (0.1-1 μM) versterkte de tweede fase van de concentratie-responscurve van 5-HT met ca. 20-40 %, terwijl vanaf 0.3 μM het de eerste fase remde. Ook in aanwezigheid van cisapride remde TTX de relaxaties geïnduceerd door cisapride volledig. Cisapride had geen effect op de concentratie-responscurven van ISO, GTN, nitroprusside of exogeen ATP, hetgeen de specificiteit van het effect van cisapride onderstreept. De 5-HT-relaxatie-versterkende effect van cisapride kon niet worden nagebootst met fentolamine, NAN-190, spiperon, citalopram, paroxetine, pargyline of SDZ 205-557. In aanwezigheid van L-NNA versterkte cisapride nog steeds de overgebleven relaxaties na toediening van 5-HT (2- tot 3-voudig). In de aanwezigheid van suramine echter versterkte cisapride de 5-HT-geïnduceerde relaxaties niet. In aanwezigheid van L-NNA kon suramine de cisapride-versterkte relaxaties met 90 % remmen.

Uit deze gegevens werd de conclusie getrokken dat cisapride selectief het suramine-gevoelige, ATP-gemedieerde, deel van de relaxaties geïnduceerd door 5-HT faciliteert via een ongeïdentificeerd effect op de intramurale zenuwen.

Een nieuwe methode voor de bestudering van peristaltiek in het colon van de geanaestheseerde cavia werd ontwikkeld (**Chapter 9**). Na laparotomie werd een rubber ballonnetje in het lumen van het distale deel van het colon ingebracht en opgeblazen met 0.05-0.2 ml water, teneinde een keutel na te bootsen. De beweging van de ballon en van de longitudinale spierlaag kon met isotonische opnemers worden gemeten. Opblazen van de ballon *per se* induceerde geen propulsie. Na behandeling met fentolamine echter (2 mg/kg i.v.) trad propulsie van de ballon op. Deze observatie suggereert een sympatische over-activiteit ten gevolge van de anaesthesie en de chirurgische handelingen, hetgeen resulteert in een tonische remmende invloed op het colon. Naloxon (1 mg/kg i.v.) alleen had geen stimulerend effect op de peristaltiek. Wanneer echter naloxon gegeven werd als toevoeging met fentolamine, dan werd een afname van de maximale, maar niet de gemiddelde propulsiesnelheid waargenomen in vergelijking met behandeling met alleen fentolamine. Behandeling met L-NNA (25 mg/kg i.v.) als toevoeging met fentolamine induceerde een toename in de gemiddelde maar niet in de maximale propulsiesnelheid vergeleken met behandeling met alleen fentolamine. De propulsiesnelheid was niet afhankelijk van het volume van de ballon, onafhankelijk van welk stoffen vooraf toegediend waren.

Dit model voorziet in de eerste methode om de effecten op propulsie van benzamiden en 5-HT-receptor-(ant)agonisten, zoals in de voorgaande hoofdstukken beschreven, te kunnen evalueren *in vivo* in de cavia.

List of publications

papers

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1992) Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*. *Br J Pharmacol* 107: 756-761.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1993) Pharmacological characterization of 5-hydroxytryptamine receptor types in the guinea-pig proximal colon. *J Gastrointest Motil* 5: 239-246.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1993) Cisapride and a structural analogue, R 76 186, are 5-hydroxytryptamine$_4$ (5-HT$_4$) receptor agonists on the guinea-pig colon ascendens. *Naunyn-Schmiedeberg's Arch Pharmacol* 347: 464-470.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1993) Substance P-induced contractions of the guinea-pig proximal colon through stimulation of post-junctional tachykinin NK$_1$ receptors. *Eur J Pharmacol* 250: 181-183.

Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA, Schuurkes JAJ (1995) 5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide. *Naunyn-Schmiedeberg's Arch Pharmacol* 351: 126-135.

Briejer MR, Akkermans LMA, Schuurkes JAJ (1995) Interactions of serotonin with multiple receptors and neurotransmitters in the guinea-pig isolated colon. *Arch Int Pharmacodyn* in press.

Briejer MR, Veen GJ, Akkermans LMA, Lefebvre RA, Schuurkes JAJ Cisapride and structural analogs selectively enhance 5-hydroxytryptamine (5-HT)-induced purinergic transmission in the guinea pig proximal colon. *J Pharmacol Exp Ther*, in press.

Briejer MR, Akkermans LMA, Lefebvre RA, Schuurkes JAJ (1995) Novel 5-HT$_2$-like receptor mediates neurogenic relaxation on the guinea-pig proximal colon. *Eur J Pharmacol* in press.

Briejer MR, Tonini M, Akkermans LMA, Schuurkes JAJ A novel *in vivo* method for the assessment of peristalsis in the distal colon of the guinea-pig. (submitted for publication)

Briejer MR, Akkermans LMA, Schuurkes JAJ On the mechanism of action of gastrointestinal prokinetic substituted benzamides. (in preparation)

Meulemans AL, Briejer MR, Helsen L, Schuurkes JAJ Characterization of the 5-HT receptor type mediating relaxation of the isolated guinea-pig stomach. (in preparation)

abstracts

Briejer MR, Meulemans AL, Schuurkes JAJ (1991) Site related distribution of 5-HT-receptors in guinea-pig colon ascendens. *Pflügers Arch Eur J Physiol* 419: R 5.

Briejer MR, Meulemans AL, Schuurkes JAJ (1991) Cisapride acts as a 5-HT_4 receptor agonist in the guinea-pig colon ascendens. *Gastroenterology* 100: A425.

Briejer MR, Meulemans AL, Lefebvre RA, Akkermans LMA, Schuurkes JAJ (1992) 5-HT-induced relaxations of guinea-pig colon mediated by nitric oxide. *J Gastrointest Motil* 4: 212

Briejer MR, Akkermans LMA, Schuurkes JAJ (1993) Novel 5-HT_1-like receptors mediate relaxations to 5-HT on the guinea-pig proximal colon. *Pharm World Sci* 15 (suppl. 1): 1a.

Briejer MR, Akkermans LMA, Schuurkes JAJ (1994) Novel 5-HT receptor type mediating neurogenic relaxation of guinea pig colon. *Behav Brain Res* (in press)

Briejer MR, Veen GJ, Akkermans LMA, Schuurkes JAJ (1994) Cisapride facilitates inhibitory purinergic transmission in the guinea-pig colon. *Gastroenterology* 106: A471.

Briejer MR, Meulemans AL, Akkermans LMA, Lefebvre RA, Schuurkes JAJ (1994) Next to NO, ATP is involved in 5-HT-induced relaxations of the guinea-pig colon as well. *Gastroenterology* 107: 1230P.

Briejer MR, Akkermans LMA, Schuurkes JAJ (1995) Orphan 5-HT receptor mediating neurogenic relaxation in guinea-pig colon. *Acta Gastro-Enterologica Belgica* 58: B11.

Dankwoord / Acknowledgements

Jan Schuurkes: Jan heeft zich altijd bijzonder ingespannen om mijn stage, en daarna mijn promotie-onderzoek, tot een buitengewoon aangename en leerzame ervaring te laten zijn, zowel op het zakelijk vlak als op privé-terrein. Jan, ik wil je bijzonder bedanken voor alles wat je mij geboden hebt. Ik ben er zeker van dat we de prettige samenwerking kunnen continueren.
Ann Meulemans: Ook zij is een zeer "plezante" persoon in de omgang (maar met een viltstift in haar hand is ze niet te vertrouwen). Ze stond altijd klaar om mij op wat voor een manier dan ook te helpen. Ann, ontzettend bedankt voor de leuke samenwerking! Ik ben blij dat ik je collega mag worden.
Louis Akkermans: Onze samenwerking is met de jaren alleen maar geïntensiveerd, culminerend in een drie-daags bezoek tijdens mijn verblijf in de Verenigde Staten. Louis, ik wil je ontzettend bedanken voor alle moeite die je je getroost hebt om dit boekje dit boekje te laten zijn, en ik hoop in de toekomst nog veel met je te maken te hebben.
Prof. Romain Lefebvre: Prof. Lefebvre is een bijzonder deskundig en bescheiden persoon, die altijd zeer consciëntieus en als eerste mijn manuscripten had nagekeken, voorzien van nuttige en altijd rechtvaardige opmerkingen en adviezen. Prof. Lefebvre, Uw bijdrage aan de inhoud van mijn proefschrift is aanzienlijk, en ik wil U hartelijk bedanken voor Uw altijd prompte reacties en adviezen. Ik hoop in de toekomst ook nog vaak met U te mogen samenwerken.
Prof. Marcello Tonini: Prof. Tonini hosted me during my two months stay in the beautiful old city of Pavia, which were a success primarily from a scientific, but also from a personal and a cultural point of view. A year later, Prof. Tonini came over to Beerse for two weeks, were we worked on the *in vivo* technique as described. Prof. Tonini, I would like to thank you for the nice and fruitful time we spend together. I also thank Barbara Balestra for her help: molto grazie.
Dr Joseph H. Szurszewski: I was given the opportunity to work for two months in the laboratory of Dr Szurszewski at the Mayo Clinic in Rochester, Minnesota (USA). Dr Szurszewski, it was an honour and a pleasure to work in your laboratory, and I would like to thank you again for your hospitality. I would also like to thank Leonid (and his wife), Bruno, Gang, Phil, Steven, Gary, Gail, Samy, Gianrico, Xiaorong, Jan and Lei.
Wouter van Berckel (alias Bulle) en Gerben Veen: De aanwezigheid van deze twee farmacie-stagiairs op de afdeling is in hoge mate op prijs gesteld door allen. Gerben en Bulle, jullie hebben veel betekend voor het verloop van mijn onder-

zoek, en jullie inzet was enorm. Ook het paranimfenwerk hebben jullie met enthousiasme aangepakt. Ge zijt toffe venten!!

Afdeling Gastro-intestinale Farmacologie: Met de stage erbij geteld heb ik 4 1/2 jaar doorgebracht op deze afdeling. Met Patrick Van Bergen (Pietje) heb ik waarschijnlijk het meest intensief contact gehad, ook na laboratorium-uren. De research-stoffenencyclopedie Etienne Ghoos is de man die altijd grote interesse had voor mijn bezigheden. Ludo Helsen toonde vooral veel interesse in mijn persoonlijke leven (niet dat de hiervoor genoemde personen dat niet deden!) en mijn Hollander-zijn, waarover hij dan ook graag een (uiteraard flauw) grapje placht te maken. Chris Borgmans verschafte het *in-vitro*-deel van de afdeling van de broodnodige vrouwelijke charme en relativering, vooral tijdens de overdoses geblaat van bovengenoemde personen. Ook Paul De Winter toonde vooral politieke en taalkundige interesse voor het Nederlandse. Over dit laatste heb ik met Jan Kuyps ook het nodige boompje opgezet. Walter De Ridder was altijd bereid te helpen met problemen van computerkundige en statistische aard. Overigens was de hulpvaardigheid van alle bovengenoemde personen geweldig. Ik wil hen en de overige leden van de afdeling, te weten Dirk Smets, Jef Ceulemans, Annemie Wellens, Jan Eelen, Eugeen Cruysbergs, Rik Luyckx en Isabelle Rutten hartelijk bedanken voor geboden hulp en hun viendelijkheid. Diane Verkuringen ben ik veel dank verschuldigd voor alle secretariële ondersteuning.

En verder: De cardiovasculairen Walter Janssens, Frans Proost, Luc Hoskens, Danny Geyskens, Jan Verrelst, Frank Cools, en iedereen van de apotheek ben ik ook erkentelijk. Lambert Leijssen, Gert Verheyen, Raymond Broeckx, Stan Eyckens, Louis Schellekens, Jan Voeten, Rene Wilems, Fons Vander Auwera, Bart Van Mengsel dank ik voor alle hulp. Paul Moerman tekende het omslag, waarvoor mijn grote waardering.

Janssen Research Foundation: Het promotie-onderzoek werd volledig uitgevoerd bij en financieel ondersteund door de Janssen Research Foundation (onderdeel van Janssen Pharmaceutica). Ik wil graag de president van de Janssen Research Foundation, Staf Van Reet, en de vice-president, Marcel Janssen, van harte bedanken voor het feit dat zij dit onderzoek mogelijk hebben gemaakt.

Beoordelingscommissie: Prof. dr P.R. Saxena, prof. dr F.P. Nijkamp en prof. dr J.H. Koeman ben ik zeer erkentelijk voor het doornemen van mijn proefschrift.

En dan: Nicole, pa en ma, en de ouders van Nicole wil ik nog noemen omdat zij altijd grote interesse hebben getoond in de voortgang van mijn bezigheden in het Belgische. Bedankt voor alle steun, belangstelling, en relativering.

Curriculum vitae

Michel Raymond Briejer werd op 23 oktober 1965 in Den Haag geboren. Na 6 jaren OVWO-onderwijs te hebben genoten aan het Huygens Lyceum te Voorburg, behaalde hij in 1984 zijn diploma. In datzelfde jaar begon hij zijn studie Scheikundige Technologie aan de Technische Hogeschool te Delft. Nadat hij zijn propedeuse had behaald besloot hij te veranderen van studie. De in 1986 begonnen studie Farmacie aan de Rijks Universiteit Utrecht werd in 1991 afgesloten met een *cum laude* gehonoreerde doctoraalbul. Als specialisaties werden de differentiatiepaketten Immunofarmacologie en Drug Design doorlopen. De stage (september 1990-maart 1991) had plaats bij de Afdeling Gastrointestinale Farmacologie van Janssen Pharmaceutica te België onder begeleiding van dr J.A.J. Schuurkes en supervisie van prof. dr F.P. Nijkamp. De scriptie, die onder begeleiding van dr A.S. Koster werd geschreven, droeg de titel *Vasoactive intestinal polypeptide: Is VIP an inhibitory neurotransmitter of the non-adrenergic, non-cholinergic nerves of the gastrointestinal tract?* Vervolgens begon hij aan een promotie-onderzoek bij de Afdeling Gastrointestinale Farmacologie van Janssen Pharmaceutica, als Assistent in Opleiding (AIO) bij Fysiologie van Mens en Dier aan de Landbouwuniversiteit in Wageningen. De cursus Proefdierkunde (als bedoeld in art. 2, tweede lid, van het Dierproevenbesluit (Stb. 1985, 336)) werd gevolgd, en afgesloten met het behalen van het certificaat. Tijdens zijn AIO-periode werkte hij in april en mei 1992 in het laboratorium van prof. dr M.Tonini (Sezione di Farmacologia e Tossicologia, Dipartimento di Medicina Interna e Terapia Medica, Universiteit van Pavia, Italië) en in oktober en november 1994 in het laboratorium van dr J.H. Szurszewski (Department of Physiology and Biophysics, Mayo Clinic, Rochester, Minnesota, USA). De auteur is per 1 juni 1995 werkzaam bij de eerder genoemde Afdeling Gastrointestinale Farmacologie als Research Associate.