An improved *in vitro* bioassay for the study of 5-HT$_4$ receptors in the human isolated large intestinal circular muscle

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1 Recently, it was demonstrated that 5-HT induces relaxation of human colon circular muscle through activation of 5-HT$_4$ receptors and 5-HT$_7$ receptors. The aim of the current study was to develop a new *in vitro* bioassay of human colon that would facilitate the pharmacological analysis of 5-HT responses mediated solely by 5-HT$_4$ receptors.

2 Contracting circular muscle strips with KCl (80 mM) yielded a stable contractile tension and, in contrast to muscarinic cholinoceptor agonists and histamine, a profound reduction of spontaneous contractility. This allowed the establishment of reproducible, fully-defined, agonist concentration-response curves by cumulative dosing. Under these conditions, 5-HT induced a concentration-dependent relaxation (pEC$_{50}$ 7.31, Hill slope 0.91).

3 Neither methysergide (10 μM) nor granisetron (1 μM) affected the 5-HT-induced relaxation, suggesting that 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-ht$_5$, 5-HT$_6$ or 5-HT$_7$ receptors are not involved. The lack of effect of tetrodotoxin (0.3 μM) indicated a direct effect of 5-HT on the smooth muscle.

4 The selective 5-HT$_4$ receptor antagonists GR 113808, GR 125487 and RS 39604 competitively antagonized the 5-HT-induced relaxation (pK$_B$ 9.43, 10.12 and 8.53, respectively). SB 204070 (1 nM) produced a rightward shift (pA$_2$ 10.34) and depression of the 5-HT curve. These affinity estimates are similar to those previously reported for 5-HT$_4$ receptors.

5 The selective 5-HT$_4$ receptor agonists, prucalopride and R076186, induced relaxations (pEC$_{50}$ 7.50 and 7.57, respectively), that were blocked by GR 113808 (3 nM), yielding pA$_2$ estimates of 9.31 and 9.21, respectively.

6 To summarise, in KCl (80 mM)-contracted muscle strips, 5-HT induces relaxation through activation of a homogeneous smooth muscle 5-HT$_4$ receptor population. This new bioassay allows the focused, pharmacological characterization of human colonic 5-HT$_4$ receptors *in vitro*.
British Journal of Pharmacology (2000) **129**, 1601–1608

Keywords: 5-HT$_4$ receptors; relaxation; 5-hydroxytryptamine; human; colon; large intestine

Abbreviations: D receptor, dibenzyline (phenoxybenzamine)-sensitive 5-HT receptor; M receptor, morphine-sensitive 5-HT receptor

Introduction

5-HT$_4$ receptors are representatives of the seven transmembrane domain, G-protein-coupled receptor family that are positively coupled to adenylyl cyclase (Dumuis *et al.*, 1988). They have been extensively studied in human tissues, including the brain (Reynolds *et al.*, 1995), heart (Kaumann, 1993), urinary bladder (Candura *et al.*, 1996), and gut (Hedge & Eglen, 1996). With respect to the upper gastrointestinal tract, 5-HT$_4$ receptors have been found in the stomach, facilitating cholinergic neurotransmission (Schuurkes *et al.*, 1991), and in the small intestine, involved in secretory processes (Borman & Burleigh, 1993) and relaxation (Kuemmerle *et al.*, 1995). Recently, it was suggested that 5-HT$_4$ receptors are involved in the initiation of the peristaltic reflex (Grider *et al.*, 1998).

In studies of human large intestine circular muscle, Burleigh (1977) observed that 5-HT induced a methysergide- and tetrodotoxin-insensitive relaxation. This 5-HT receptor was called 'atypical', as it could not be classified contemporarily as

either the M receptor (Morphine-sensitive 5-HT receptor, corresponding to the tetrodotoxin-sensitive 5-HT$_3$ receptor) or D receptor (Dibenzyline (phenoxybenzamine)-sensitive 5-HT receptor, corresponding to the methysergide-sensitive 5-HT$_2$ receptor). Subsequently, the 5-HT-induced relaxation of the circular muscle was ascribed to smooth muscle 5-HT$_4$ receptor stimulation (Tam *et al.*, 1994; McLean *et al.*, 1995; Meulemans *et al.*, 1995). Additionally, it was demonstrated that the activation of 5-HT$_4$ receptors in the human colon was associated with the stimulation of cyclic AMP formation (McLean & Coupar, 1996).

These *in vitro* studies have contributed to the current knowledge of the involvement of 5-HT$_4$ receptors in the motility of the lower gastrointestinal tract. Selective 5-HT$_4$ receptor agonists, such as prucalopride (Emmanuel *et al.*, 1998; Briejer *et al.*, 1998a) and SDZ HTF-919 (Appel *et al.*, 1997a,b), have been demonstrated to facilitate colonic transit and increase stool frequency. In this manner, selective 5-HT$_4$ receptor agonists might be used to treat idiopathic constipation (Briejer *et al.*, 1999). On the other hand, it was proposed that selective 5-HT$_4$ receptor antagonists could be applied to relieve patients suffering from irritable bowel syndrome,

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presumably by reducing the hypersensitive response to endogenously released 5-HT at $5-HT_4$ receptors in these patients (Sanger, 1996).

However, in *in vitro* assays of human colon, selective competitive $5-HT_4$ receptor antagonists did not produce simple, concentration-dependent competitive antagonism of 5-HT-induced relaxation, either in studies measuring direct relaxation (McLean & Coupar, 1995; Meulemans *et al.*, 1995) or the inhibition of spontaneous contractility (Tam *et al.*, 1995). It has recently been reported that this complexity is due to the concomitant stimulation of $5-HT_7$ receptors, for which the selective $5-HT_4$ receptor antagonists have negligible affinity (Prins *et al.*, 1999b). In addition to this receptor heterogeneity, the pharmacological characterization of the $5-HT_4$ receptors mediating smooth muscle relaxation is further complicated by the presence of spontaneous contractility that precludes the reliable measurement of 5-HT responses under existing assay conditions. Here, we report our attempts to improve the bioassay of human colon muscle to allow selective quantitative analysis of the $5-HT_4$ receptor-mediated component of 5-HT-induced relaxation.

Methods

Preparation

With the approval of the local ethics committee, segments of colon (ascending to sigmoid) and rectum were obtained from patients undergoing surgery for colonic cancer. The segment of colon was cut open longitudinally and luminal contents were rinsed out with Krebs-Henseleit solution (containing (mM) glucose 11.1, $CaCl_2$ 2.51, $NaHCO_3$ 25, $MgSO_4$ 1.18, KH_2PO_4 1.18, KCl 4.69 and NaCl 118) and the mucosa and mesentery were removed. The preparations were stored overnight at $4°C$ in fresh Krebs-Henseleit solution. The next day, eight circular muscle strips of approximately $2-3$ cm were cut from the intertaenial area. The strips were anchored to organ bath hooks and suspended in a classical organ bath set-up for isometric measurement (20 mN tension). The 20 ml organ baths were filled with Krebs-Henseleit solution, kept at $37°C$ and gassed with carbogen (95% O_2, 5% CO_2).

Experimental protocol

The organ bath solution was replaced every 15 min for at least 60 min, until spontaneous contractility occurred. Then, treatment or vehicle were administered and left to incubate for 60 min. When included, pargyline (0.1 mM) was incubated for 30 min followed by a double replacement of the organ bath solution and a further period of 30 min. After the incubation, the strips were contracted with KCl (80 mM) and, after a stable contraction had been established (after approx. $120-180$ min), agonists were added to the organ bath solution by cumulative dosing in half log unit concentration increments beginning at 1 nM. Only one curve was obtained per strip and at least one control curve to 5-HT was established per specimen of colon or rectum obtained. Approximately 16% of the total number of colon strips prepared were precluded from analysis for various reasons, such as inability to contract to KCl (inertia), decay of KCl-induced contraction and strips torn off the organ hook during contraction to KCl. Although no information was available to us concerning the state/pathology of each specimen there did not appear to be a relationship between those variables that were known to us (patient age, sex, and indication for surgery) and the failure of strips to respond.

Data analysis

The effect of compounds used to pre-contract the tissue on spontaneous contractility was expressed as the ratio of the amplitude of spontaneous contractility for a period of 5 min prior to contraction (X) over the amplitude of spontaneous contractility for 5 min prior to the addition of any relaxatory agonist (Y). In order to provide an estimate of this ratio when KCl was used as the pre-contractile agent, tracings of one muscle strip from each of six different specimens were chosen at random.

Responses to 5-HT receptor agonists were expressed as percentage of the within preparation KCl (80 mM)-induced contraction for both analysis and graphical presentation. Individual cumulative concentration-relaxation curves to agonists were fitted to the Hill equation, obtaining estimates for midpoint location pEC_{50}, Hill slope (n_H) and maximum asymptote (α). The effect of pre-treatment on these parameters was tested by one-way ANOVA ($P < 0.05$). Antagonist affinity estimates were obtained by an iterative fitting procedure using the Schild equation, providing an estimate for pK_B (according to the method described by Black *et al.*, 1985).

If only one concentration of antagonist was tested, and the agonist curve was shifted to the right with no change in upper asymptote or slope, the antagonist affinity was expressed as a pA_2 value calculated using the Schild equation. When an antagonist produced a depression of the curve, in addition to a rightward shift, the antagonist potency was expressed as an apparent pA_2 value, also using the Schild equation (Arunlakshana & Schild, 1959).

Statistical analysis

To test the criteria for Schild-analysis, one-way ANOVA was performed, followed by a *post-hoc* Bonferroni's test for multiple comparisons. The effect of a single pretreatment was assessed by one-way ANOVA. A level of $P < 0.05$ was considered to be significant. As some strips were precluded from subsequent analysis (see above), the number of patient tissues (denoted by *n*) in the control group was sometimes different from that in the treatment groups.

Compounds

The following compounds were used (with their respective suppliers given in parentheses): (1-butyl-4-piperidinyl)-methyl-8-amino-7-chloro1,4-benzodioxane-5-carboxylate HCl (SB 204070), 1-[2-[(methylsulphonyl)amino] ethyl]-4-piperidinyl-methyl 5-fluoro-2-methoxy-1H-indole-3-carboxylate (GR 125487), [1-[2-[(methylsulphonyl)amino]ethyl]-4-piperidinyl]-methyl 1-methyl-1H-indole-3-carboxylate (GR 113808), granisetron HCl, 4-amino-5-chloro-2,3-dihydro-N-(1-[3-methoxypropyl]-4-piperidinyl)-7-benzofurancarboxamide HCl (prucalopride; R093877), cis-4-amino-5-chloro-N-[1-[4-[4-(dimethylamino)-1-piperidinyl]-4-oxo-butyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide (R076186) (Janssen Research Foundation, Belgium); carbachol, histamine HCl (Janssen Chimica, Belgium); tetrodotoxin, 5-HT creatinine sulphate, (Serva, Germany); methysergide maleate (Sandoz, Switzerland); pargyline HCl (Abbott, U.S.A.); potassium chloride (KCl; Sigma, Belgium); corticosterone, cocaine HCl (Merck, Germany); fluoxetine HCl (Tocris Cookson, U.K.); 5-methylfurmethide (James Black Foundation, U.K.) and 1-(4-amino-5-chloro-2-(3,5-dimethoxy) benzyloxyphenyl)-3-[1-((2-methylsulphonylamino)ethyl)piperidin-4-yl]-1-propanone (RS 39604; Merck Belgolabo, Belgium).

All compounds were dissolved in 0.9% NaCl solution, except for GR 113808 and R076186, which were dissolved in 0.9% NaCl acidified in the stock solution with tartaric acid, and pargyline, which was dissolved in distilled water with 10% cyclodextrin in the stock solution. The solvents had no effect on the baseline tension or the curves to 5-HT, and the total volume of compound solution added to the organ bath never exceeded 1 ml, to avoid significant dilution of the KCl concentration. The solutions were prepared freshly on the day of the experiment and all dilutions were prepared using 0.9% NaCl solution.

Results

In order to study relaxations to low concentrations of agonists and to construct reproducible concentration-relaxation curves, a stable basal contractile state of the muscle strips is required. The spontaneous contractility, that progressively developed while replacing the organ bath solution every 15 min, was subject to variability both in amplitude and frequency. This variation was observed within one strip, between the strips of one specimen, and also between the strips of different specimens. Accordingly, the spontaneous contractility was not used as a contractile state from which to measure relaxatory responses to 5-HT and further preliminary experimentation was performed in order to obtain reproducible and stable precontractions. The non-selective muscarinic cholinoceptor agonists, carbachol (1 μM, spontaneous contractility ratio 0.8 ± 0.1; $n = 6$

specimens) and 5-methylfurmethide (1 μM, ratio 0.4 ± 0.2; $n = 2$ specimens), produced stable increases in contractile tension but they also enhanced spontaneous contractions decreasing the assay signal-to-noise ratio. Histamine (10 μM, ratio 0.9 ± 0.3; $n = 2$ specimens) had a similar effect and was thus also considered unsuitable for these studies. In contrast, KCl (80 mM) induced a contraction that reached an initial peak within about 30 min and then faded to a variable extent over 120–180 min to attain a plateau. This was maintained for at least a further 120 min, with reduced spontaneous contractility (Figure 1; ratio 16 ± 7). As a consequence, KCl was chosen to contract the strips in all further experiments.

5-HT induced a concentration-dependent relaxation of KCl contracted muscle strips, yielding a monophasic sigmoidal concentration-response curve (Figure 1 and Table 1), consistent with a single-site interaction. The 23 specimens used in the experiments were distributed down the large intestine as follows; ascending colon (four specimens), transverse colon (one), descending colon (one), sigmoid colon (16) and rectum (one). The midpoint location, slope and upper asymptotes of the 5-HT curves obtained in the two regions for which there sufficient samples to make a formal comparison, the ascending colon, ($pEC_{50} = 7.34 \pm 0.10$, $n_H = 0.92 \pm 0.16$, $\alpha = 44 \pm 7\%$) and the sigmoid colon ($pEC_{50} = 7.35 \pm 0.05$, $n_H = 1.06 \pm 0.11$, $\alpha = 31 \pm 3\%$), were indistinguishable. Similarly, there were no other obvious differences in the 5-HT curves obtained on tissues from the other regions. Accordingly, the data from all regions were pooled for subsequent analysis.
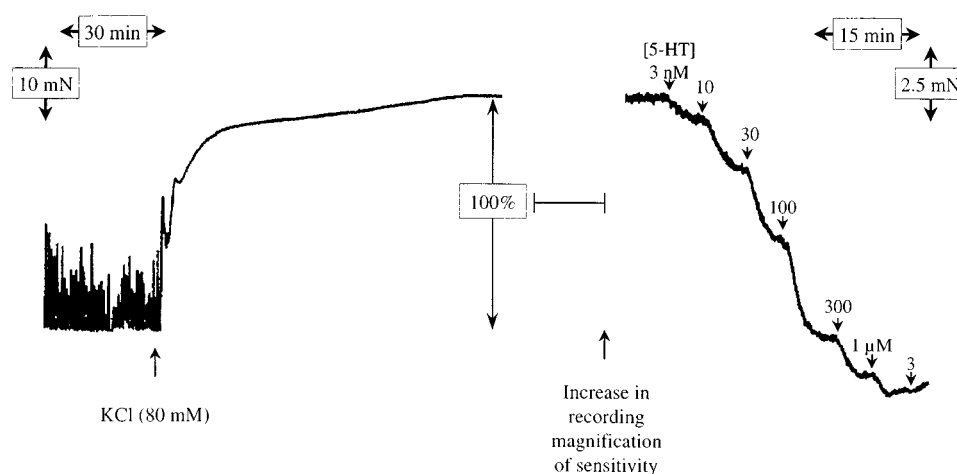

Figure 1 Representative chart-recorder tracing of an experiment with human isolated colonic circular muscle. Shown are the KCl-induced contraction (left), which, after a stabilization time and an increase in the recording magnification of sensitivity, is followed by adding 5-HT in half log unit ascending cumulative concentrations (right).

Table 1 Effects of tetrodotoxin, receptor antagonists and inhibitiors of re-uptake and breakdown, on mean curve parameters of 5-HT in human colon circular muscle assay

Compound	Concentration	n	pEC_{50}	n_H	α (%)
Control		8	7.32 ± 0.07	0.88 ± 0.08	41.3 ± 4.6
Pargyline	0.1 mM	6	7.42 ± 0.04	1.14 ± 0.11	$25.8 \pm 5.2^*$
Cocaine	30 μM	5	7.18 ± 0.14	1.02 ± 0.12	50.7 ± 10.0
Corticosterone	30 μM	6	7.26 ± 0.07	0.93 ± 0.09	27.3 ± 6.0
Fluoxetine	1 μM	5	7.49 ± 0.09	0.97 ± 0.08	34.7 ± 10.0
Tetrodotoxin	0.3 μM	6	7.34 ± 0.10	0.89 ± 0.12	48.4 ± 9.6
Granisetron	1 μM	6	7.29 ± 0.06	1.01 ± 0.10	40.6 ± 9.3
Methysergide	10 μM	6	7.54 ± 0.13	0.87 ± 0.09	48.3 ± 8.4

*indicates significant difference from control ($P < 0.05$).

Inhibitors of uptake-1 (cocaine, 30 μM), uptake-2 (corticosterone, 30 μM) or selective serotonin re-uptake (fluoxetine, 1 μM) had no significant effect on the 5-HT curve parameters. Irreversible and non-selective inhibition of monoamine oxidase by pargyline (0.1 mM) significantly reduced the maximum response to 5-HT without increasing the potency. Hence, inhibitors of breakdown or re-uptake of 5-HT were not used. Tetrodotoxin (0.3 μM) did not affect the curve to 5-HT, neither did it change the precontraction induced by KCl. The data relating to the effects of the above-mentioned compounds on the 5-HT-induced relaxation are presented in Table 1.

Agonists

5-HT and the selective 5-HT$_4$ receptor agonists, prucalopride and R076186, produced concentration-dependent relaxations
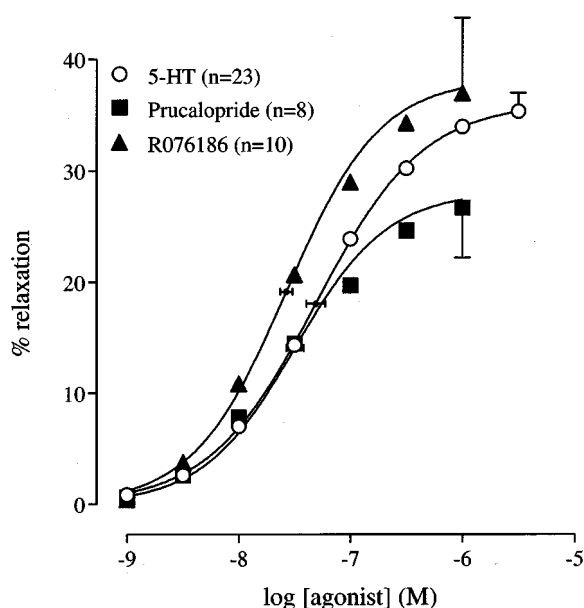

Figure 2 Concentration-response curves to 5-HT, prucalopride and R076186 in the human isolated colonic circular muscle. The curves shown superimposed on the mean experimental data points represent simulations using the Hill-equation and the parameters for midpoint location (with horizontal standard error bars), upper asymptote location (with vertical standard error bars) and the Hill slope, that were obtained from the iterative fitting procedure.

(Figures 1 and 2, Table 2). The midpoint location, slope and upper asymptote estimates for the 5-HT, prucalopride and R076186 curves were not significantly different.

Antagonists

The selective 5-HT$_3$ receptor antagonist granisetron (1 μM; Sanger & Nelson, 1989) did not alter the curve to 5-HT, indicating that 5-HT$_3$ receptors were not involved (Table 1). Similarly, the 5-HT$_1$, 5-HT$_2$, 5-ht$_5$, 5-HT$_6$, 5-HT$_7$ receptor antagonist methysergide (10 μM; Gommeren *et al.*, 1998) did not affect the curve to 5-HT. None of the selective 5-HT$_4$ receptor antagonists GR 113808, GR 125487 and RS 39604 significantly affected the maximal response or the Hill slope of the 5-HT curve and thus they all behaved as simple competitive antagonists (pK$_B$ 9.43$\pm$0.07; 10.12$\pm$0.07 and 8.53$\pm$0.10, respectively; Figure 3; Table 2). Another selective 5-HT$_4$ receptor antagonist, SB 204070, was only tested at 1 nM. This concentration shifted the 5-HT curve to the right (apparent pA$_2$=10.34$\pm$0.11) but also reduced the maximal response to 5-HT (Figure 4). Additionally, GR 113808 (3 nM) produced a parallel rightward shift of prucalopride and R076186 curves associated with pA$_2$ values of 9.31$\pm$0.14 and 9.21$\pm$0.12, respectively (Figure 5).

Discussion

The present data indicate that when KCl (80 mM) is used to pre-contract human colonic circular smooth muscle, the relaxation induced by 5-HT is mediated solely by a homogeneous population of 5-HT$_4$ receptors. Prior to this study, relaxations to 5-HT were assessed in bioassays measuring inhibition of spontaneous contractility (Tam *et al.*, 1994), relaxation of tone either in the absence or presence of carbachol (McLean *et al.*, 1995) or relaxation of histamine-induced contractions (Meulemans *et al.*, 1995). However, in our hands, it was this spontaneous contractility, and, moreover, the lack of a stable tone that complicated the establishment of reproducible cumulative concentration-response curves. The use of KCl minimized the spontaneous contractility and induced a slowly equilibrating but stable tone so that reproducible, fully-defined, agonist concentration-response curves could be obtained by cumulative dosing. It has been demonstrated that KCl-induced contractions are suitable for the observation of relaxations to various agonists

Table 2 5-HT$_4$ receptor agonist parameters (pEC$_{50}$, n$_H$, and α) and antagonist parameters (pK$_B$/pA$_2$) in the human colon circular muscle assay

Compound	n	pEC$_{50}$	n$_H$	α (%)	Tam *et al.* (1994)	McLean *et al.* (1995)
Agonists						
5-HT	23	7.31$\pm$0.08	0.91$\pm$0.13	36.1$\pm$1.6	6.7	6.8
Prucalopride	8	7.50$\pm$0.08	1.05$\pm$0.29	28.1$\pm$4.5		
R076186	10	7.57$\pm$0.06	1.04$\pm$0.24	38.2$\pm$6.7		
Antagonists		pK$_B$/pA$_2$	b[a]			
GR 113808	8–10	9.43$\pm$0.07	1.07$\pm$0.08		7.8–8.9[c]	8.1–9.0[d]
GR 125487	4–7	10.12$\pm$0.07	1.04$\pm$0.07			
RS 39604	6	8.53$\pm$0.10	1.15$\pm$0.14			
SB 204070	7	10.34$\pm$0.11[b]	–			

[a]The Schild plot slope parameter estimates (b) were obtained from the unconstrained fit to the modified Schild equation; the pK$_B$ estimates for GR 113808, GR 125487, RS 39604 of 5-HT-induced relaxation were obtained with the Schild slope constrained to unity. [b]SB 204070 was only tested 1 nM, yielding a rightward shift and a depression of the curve to 5-HT, therefore, a pA$_2$ value was estimated. [c]pA$_2$ values estimated at each increasing concentration of GR 113808 (3 to 100 nM) decreased from 8.9 to 7.8. [d]pA$_2$ estimates decreased from 9.0 to 8.1 with increasing concentrations of GR 113808 (10 nM to 1 μM).
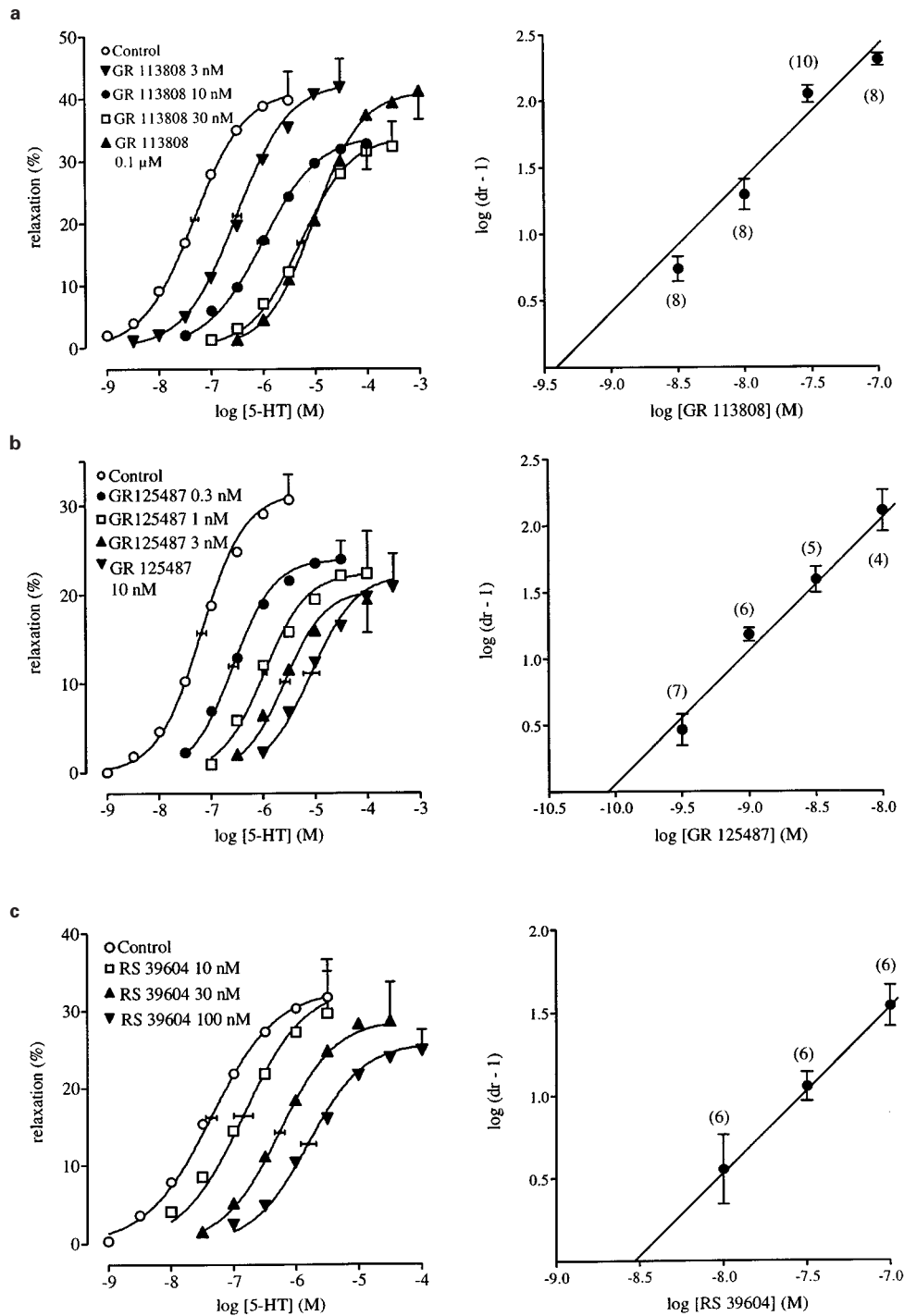
Figure 3 Left panel: The effect of (a) GR 113808 (b) GR 125487 and (c) RS 39604 on 5-HT-induced relaxation in the human isolated colonic circular muscle. The curves shown superimposed on the mean experimental data points represent simulations using the Hill-equation and the parameters for midpoint location (with horizontal standard error bars), upper asymptote location (with vertical standard error bars) and the Hill slope, that were obtained from the iterative fitting procedure. Right panel: Schild plots of GR 113808 (a); GR 125487 (b) and RS 39604 (c) with the Schild slope constrained to unity. The number depicted in parentheses above each average log (dr-1) point represents the number of curves constructed with that concentration of antagonist.

in tissues, including the bladder (Hedge *et al.*, 1997), stomach (Molderings *et al.*, 1998) and colon (MacDonald *et al.*, 1996).

Tetrodotoxin did not alter the relaxation to 5-HT, indicating that the relaxant 5-HT$_4$ receptor is located on the smooth muscle. Furthermore, fluoxetine, cocaine, corticosterone and pargyline did not alter the potency of 5-HT (although pargyline reduced the maximal response to 5-HT), demonstrating that the exogenously administered 5-HT was not subject to significant pre-junctional (re-)uptake or metabolism. If pargy-

line was producing an effect on 5-HT disposition, then a leftward shift of the midpoint location of 5-HT curve might be expected rather than the depression of the upper asymptote which was obtained. Therefore, this effect of pargyline was probably not due to an effect on inhibition of monoamine oxidase or 5-HT uptake.

The selective 5-HT$_4$ receptor agonists, prucalopride (Briejer *et al.*, 1998b) and R076186 (Briejer *et al.*, 1993) induced relaxation of the muscle strips at 5-HT$_4$ receptor-selective

concentrations. Previously, we showed that prucalopride and R076186 also induce 5-HT$_4$ receptor-mediated relaxation of canine rectal circular smooth muscle (Prins *et al.*, 1999a). The small standard errors associated with the pEC$_{50}$ estimates of all of the agonists tested, either in the absence or presence of antagonists, illustrates the improved reproducibility and increased signal-to-noise ratio in the new assays compared to those used previously (Tam *et al.*, 1994; McLean *et al.*, 1995).

The affinity estimates obtained for the selective 5-HT$_4$ receptor antagonists GR 113808 (Gale *et al.*, 1994b; Grossman *et al.*, 1993; Kaumann, 1993), GR 125487 (Gale *et al.*, 1994a), RS 39604 (Hedge *et al.*, 1995) and SB 204070 (Wardle *et al.*,

1994) were similar to previously reported values obtained in human, rat and/or guinea-pig tissues (see corresponding references). The antagonist potency for GR 113808 was independent of the agonist used since the pA$_2$ values estimated from the interaction with prucalopride and R076186 were indistinguishable from the pK$_B$ value estimated using 5-HT as agonist. The insurmountable antagonism expressed by SB 204070 is consistent with previous reports of its behaviour as a slowly dissociating antagonist in assays of guinea-pig, rat and canine tissues (Wardle *et al.*, 1994; Zeitung *et al.*, 1998; Leung *et al.*, 1995; Prins *et al.*, 1999a). In these studies, similar apparent pA$_2$ values were reported, varying between 10 and 11.

The striking observation of this study was that the 5-HT-induced relaxation appeared to be entirely mediated by 5-HT$_4$ receptor stimulation, as was demonstrated by the concentration-dependent rightward shift of the curve to 5-HT by GR 113808, GR 125487 and RS 39604 (Figure 3) over dose ratios greater than 100. The results with GR 113808 contrast with previous reports in the human colon, measuring inhibition of spontaneous contractility, in which the profile of GR 113808-induced antagonism was not consistent with competition at a single class of receptor. Thus, GR 113808 shifted the curve to 5-HT in parallel to the right at 3 nM (pA$_2$ of 8.9), however, at 10–100 nM GR 113808 produced little to no further rightward shift (Tam *et al.*, 1995; Table 2). Similarly, McLean *et al.* (1995), measuring direct relaxation, demonstrated that GR 113808 (10 nM) produced a parallel rightward displacement of the curve to 5-HT (pA$_2$ 9.0), but that this displacement did not linearly increase with the concentration of GR 113808 (100 nM; pA$_2$ 8.7 and 1 μM; pA$_2$ 8.1). In line with these observations, McLean & Coupar (1995) demonstrated that the selective 5-HT$_4$ receptor antagonist SB 207710 shifted the curve to 5-HT rightward, but the Schild slope (0.5) was significantly less than unity.

These reported deviations from expected competitivity of the selective 5-HT$_4$ receptor antagonists, pointed to the possibility of simultaneous stimulation of another 5-HT receptor for which they have lower affinity. Interestingly, in the combined presence of methysergide and ondansetron (both at 10 μM; a treatment that will not produce significant block of 5-HT$_4$ receptors), the Schild slope of SB 207710 changed to a
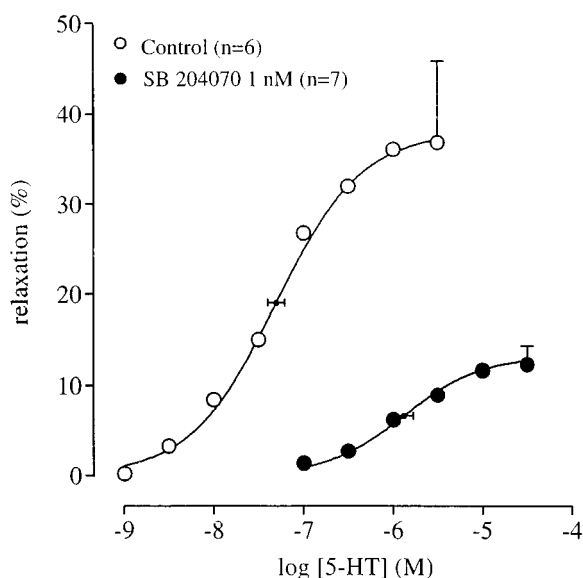

Figure 4 The effect of SB 204070 on 5-HT-induced relaxation of human isolated colonic circular muscle. The curves shown superimposed on the mean experimental data points represent simulations using the Hill-equation and the parameters for midpoint location (with horizontal standard error bars), upper asymptote location (with vertical standard error bars) and the Hill slope, that were obtained from the iterative fitting procedure.
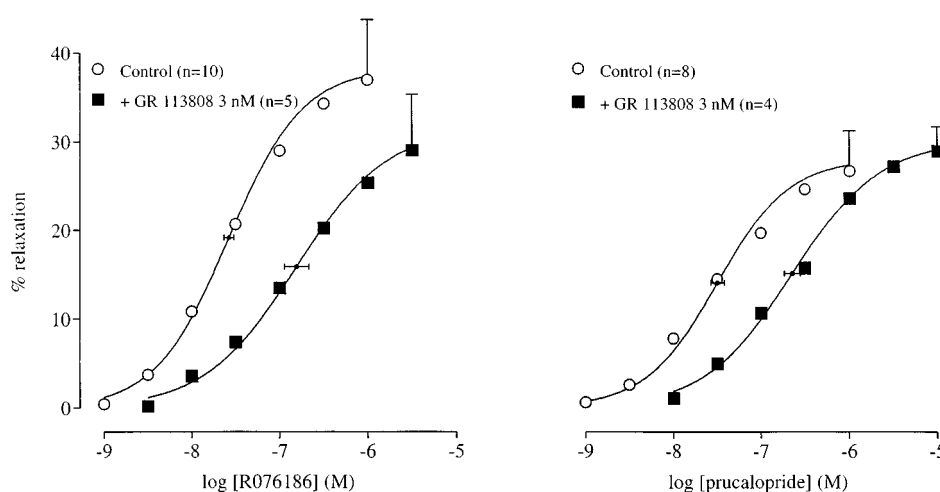

Figure 5 The effect of the selective 5-HT$_4$ receptor antagonist GR 113808 on the relaxation curve to prucalopride and R076186 in the human isolated colonic circular muscle. The curves shown superimposed on the mean experimental data points represent simulations using the Hill-equation and the parameters for midpoint location (with horizontal standard error bars), upper asymptote location (with vertical standard error bars) and the Hill slope, that were obtained from the iterative fitting procedure.

value not significantly different from unity, consistent with simple competitive antagonism (McLean & Coupar, 1995). The pK$_B$ value obtained (pK$_B$ 10.1) was consistent with those observed at 5-HT$_4$ receptors on guinea-pig colon (apparent pA$_2$ 10.0; Brown *et al.*, 1993) and human atrium (pK$_B$ 10.0; Kaumann *et al.*, 1994). An explanation of this phenomenon was found very recently, in a study in our laboratory, demonstrating that in addition to 5-HT$_4$ receptors, smooth muscle 5-HT$_7$ receptors also mediate relaxation of human colon (Prins *et al.*, 1999b). Therefore, it is most likely that the potent 5-HT$_7$ receptor antagonist methysergide (Carter *et al.*, 1995; Gommeren *et al.*, 1998) was inhibiting the effect of 5-HT$_7$ receptors in the study by McLean & Coupar (1995).

The bioassay developed here encounters none of these problems, as the necessity to include antagonists to observe 5-HT$_4$ receptor-mediated effects alone is absent, as shown by the parallel rightward shift by GR 125487, GR 113808 and RS 39604 of the curve to 5-HT. Presumably, the 5-HT$_7$ receptor-mediated component is sensitive to the nature and the level of pre-contraction. Why the 5-HT$_7$ receptor is not operational when the tissues are contracted with KCl, remains to be determined. The advantage of this new bioassay is that it facilitates the clear-cut, quantitative pharmacological analysis of 5-HT$_4$ receptors in the human colon. Furthermore, the minimization of spontaneous contractility allows the observation of agonism at low agonist concentrations and may increase the probability of observing responses to low-efficacy agonists.

In conclusion, it has been demonstrated that in KCl-precontracted human colonic circular muscle strips, the 5-HT-induced relaxation is due to smooth muscle 5-HT$_4$ receptor stimulation. The 5-HT-induced relaxation is exclusively mediated by 5-HT$_4$ receptors, with no involvement of 5-HT$_7$ receptors, that presumably complicated previous characterizations of colonic 5-HT$_4$ receptors. Thus, this improved bioassay allows the quantitative, pharmacological characterization of human colonic 5-HT$_4$ receptors.

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