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SPECIAL REPORT

Evidence for 5-HT_7 receptors mediating relaxation of human colonic circular smooth muscle

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5-HT_4 receptors mediate relaxation of human colon circular muscle. However, after 5-HT_4 receptor blockade (SB 204070 10 nM), 5-HT still induced a relaxation (pEC_{50} 6.3). 5-HT_4 receptors were sufficiently blocked, as the curves to 5-HT obtained in the presence of 10 and 100 nM SB 204070 were indistinguishable. This 5-HT-induced relaxation was tetrodotoxin-insensitive, indicative of a smooth muscle relaxant 5-HT receptor. This, and the rank order of potency (5-CT = 5-MeOT = 5-HT) suggested involvement of 5-HT_1 or 5-HT_7 receptors. Mesulergine, a 5-HT_7 receptor antagonist at nanomolar concentrations, and a 5-HT_1 receptor antagonist at micromolar concentrations, competitively antagonized the 5-HT-induced relaxation (pK_B 8.3) and antagonized the relaxation to 5-CT. Methysergide antagonized the 5-HT-induced relaxation (pA_2 7.6). It is concluded that the profile of the smooth muscle inhibitory 5-HT receptor resembles that of the 5-HT_7 receptor. These data provide the first evidence for functional human 5-HT_7 receptors.

Keywords: 5-HT_7 receptors; 5-HT; human; colon; circular muscle

Abbreviations: CCh, carbachol; MES, mesulergine

Introduction In studies to investigate 5-HT-induced responses in circular colonic muscle strips, it was demonstrated that 5-HT_4 receptors mediate relaxation (Tam *et al*., 1995: 5-HT_4 receptors mediating inhibition of spontaneous contractility; McLean & Coupar, 1995: 5-HT_4 receptors mediating direct relaxation). However, the 5-HT-induced relaxation could not be ascribed to a homogeneous 5-HT_4 receptor population. The selective 5-HT_4 receptor antagonist GR113808 shifted the response curve to 5-HT rightward at nanomolar concentrations, but at higher concentrations, GR113808 did not shift the curve to 5-HT further, which was reflected in a decrease in pA_2 estimates with increasing concentrations of antagonist (McLean *et al*., 1995; Tam *et al*., 1995). This suggests that the 5-HT-induced relaxation also involved a non-5-HT_4 receptor mechanism.

Interestingly, we found that in the presence of 5-HT_4 receptor blockade (induced by SB 204070), 5-HT was still able to relax the carbachol-contracted tissue and in the current study we describe the characterization of this non-5-HT_4 receptor-mediated relaxation of human colonic circular muscle.

Methods Segments of ascending, transverse, descending and sigmoid colon and rectum were obtained from patients that underwent surgery for colonic cancer (with the approval of the local Ethics Committee). The segment of colon was cut open longitudinally and luminal contents were rinsed out with modified 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 Krebs-Henseleit solution contained SB 204070 (10 nM) throughout the experiments to block 5-HT_4 receptors, with the exception of one experiment, that was carried out in the presence of methysergide (1 μM), to demonstrate the 5-HT_4 receptor-mediated component of the relaxation. From the intertaenial region, circular muscle strips of approximately 2–3 cm length and 2–3 mm width were cut. The strips were anchored to organ bath hooks and suspended in a classical organ bath set-up for isotonic measurement (2 g load). The baths were filled with Krebs solution, gassed with carbogen (95% O_2, 5% CO_2) and kept at 37°C. After set-up, the strips were left to stabilize for a period of 30 min, following which two sequences of single-dose administrations in each strip were performed; one to evoke priming relaxations to 5-HT (10 μM), which was followed by one to construct concentration-response curves to 5-HT or a tryptamine analogue. The priming sequence was initiated by precontraction with carbachol (CCh, 0.15 μM), which, after 15 min, was followed by addition of 5-HT (10 μM) to relax the tissue. After the maximum response to 5-HT had been reached, the organ bath solution was replaced twice. Thereafter, a period of 5 min of stabilization was followed by a third replacement of the organ bath solution. A period of 10 min of stabilization was allowed before the priming sequence was repeated (four times). As the relaxation to 5-HT (10 μM) rapidly reached its maximum but also rapidly declined, it was not feasible to construct curves in a cumulative manner. Therefore, a non-cumulative dosing cycle was chosen to construct curves to agonists, as is outlined in the following dosing sequence: Antagonist or vehicle was administered and left to equilibrate for 10 min, followed by addition of carbachol (0.15 μM) to the organ bath to contract the tissue. After 15 min the response to carbachol was stable, and a single dose of a 5-HT receptor agonist was added. After the maximum relaxation to the agonist was obtained, the organ bath solution was replaced twice and left to equilibrate for 5 min. Then, the organ bath solution was replaced for the third time and the dosing sequence was repeated applying the next concentration of a 5-HT receptor agonist (starting at

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10 nM, progressing at half-log unit increments). One curve was obtained per strip and one strip served to provide a control curve to 5-HT per specimen of large intestine obtained. Each response to the dose of carbachol (0.15 μM) added to contract the tissue was taken as 100% to normalize the agonist-induced relaxation of that specific carbachol-induced contraction.

Data analysis and statistics

Agonist parameters (mid-point location pEC_{50}, maximum relaxation α and Hill slope n_H) and antagonist parameters (pK_B, or pA_2) were estimated as described previously (Black *et al.*, 1985). When the treatment did not affect the shape of the curve to 5-HT, any shift was presented as ΔpEC_{50}, which was estimated as the absolute value of the average $pEC_{50,\ control}$ − the average $pEC_{50,\ treatment}$. Results were expressed in terms of mean curve parameters ± s.e.mean, and mean original data ± s.e.mean, when curve fitting was not possible. Analysis of variance (ANOVA) was performed, followed by a *post hoc* Bonferroni's test for multiple comparisons or a Student's *t*-test in the case of a single treatment. A level of $P<0.05$ was considered to indicate a statistical significant difference. The number of experiments carried out is denoted by *n*.

Compounds

The following compounds were used (with their abbreviations, if any, in italics, and respective suppliers between brackets): (1-butyl-4-piperidinyl)methyl-8-amino-7-chloro-1,4-benzodioxane-5-carboxylate HCl (SB 204070), granisetron HCl, mesulergine HCl, 5-methoxytryptamine (5-*MeOT*), 2-methyl-5-HT (2-*Me-5-HT*) (Janssen Research Foundation, Belgium), carbachol (*CCh*; Merck, Germany), methysergide maleate (Sandoz, Switzerland), tetrodotoxin, 5-hydroxytryptamine creatinine sulphate (5-*HT*, Serva, Germany), 5-carboxamidotryptamine maleate (5-*CT*; Tocris Cookson, U.K.).

The compounds were dissolved in distilled water. Distilled water (1 ml) had no effect on the concentration-response curve. All solutions were prepared freshly on the day of the experiment.

Results In the presence of methysergide (1 μM; 5-HT_1, 5-HT_2, 5-ht_5, 5-HT_6 and 5-HT_7 receptor antagonist), 5-HT induced a relaxation of carbachol (0.15 μM)-induced contractions (Figure 1A) that was sensitive to 5-HT_4 receptor blockade (10 nM SB 204070; Figure 1B). Under 5-HT_4 receptor-blocking conditions in the absence of methysergide, 5-HT still induced a relaxation (Figure 1C). From this point, the relaxation to 5-HT and the tryptamine analogues tested in the current study, were tested in the presence of SB 204070 (10 nM). Concentration-response curves to 5-HT were sigmoidal (pEC_{50} 6.3 ± 0.2; Figure 2). The 5-HT analogues 5-MeOT, 5-CT and 2-Me-5-HT all induced relaxations yielding the following rank order of agonist potency (with concomitant pEC_{50} values between parentheses): 5-HT (6.3 ± 0.2) = 5-MeOT (6.3 ± 0.2) = 5-CT (6.3 ± 0.2) > 2-Me-5-HT (5.3 ± 0.1) (Figure 2). The curves to the four agonists tested displayed maximum responses that were not significantly different from each other. The curve to 2-Me-5-HT was relatively steep (Hill slope parameter 1.7 ± 0.3) as compared to 5-HT (0.9 ± 0.1), 5-MeOT (0.8 ± 0.1) and 5-CT (0.8 ± 0.1).

Mesulergine, at concentrations of 30, 100 and 300 nM, competitively antagonized the 5-HT-induced relaxation (Figure 3), yielding a Schild slope of 0.9 ± 0.2 (not significantly different from unity) and a pK_B estimate of 8.3 ± 0.1. Mesulergine (100 nM) also significantly inhibited the 5-CT-induced relaxation (Student's *t*-test, $P<0.05$), but the maximum response to 5-CT in the presence of mesulergine was not obtained. Therefore, curve fitting could not be performed on these data (Figure 2). By inspection, the antagonism appeared insurmountable. Furthermore, the increased relaxation at the highest concentration of 5-CT administered (30 μM) may be indicative of a second phase. Methysergide (100 nM) shifted the curve to 5-HT rightward in a parallel manner (pA_2 7.6, Figure 4).

The curve to 5-HT was affected neither by tetrodotoxin (0.3 μM; ΔpEC_{50} 0.04; $n=6$; data not shown) nor by a 10 fold increase of the concentration of SB 204070 (to 100 nM; ΔpEC_{50} 0.04; $n=6$; data not shown). The selective 5-HT_3 receptor

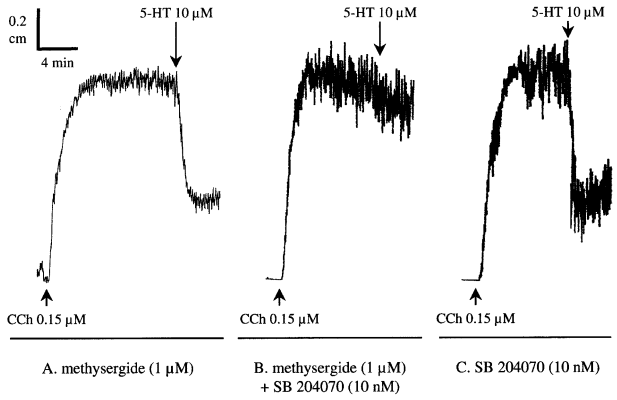

Figure 1 Chart recorder tracings of 5-HT (10 μM)-induced effects on CCh (0.15 μM)-contracted circular muscle strips of human sigmoid colon. 5-HT induces a relaxation in the presence of methysergide (1 μM; A) that was sensitive to 5-HT_4 receptor blockade by SB 204070 (10 nM; B). When SB 204070 (10 nM) was used only, 5-HT still induced a relaxation of the muscle strips (C).

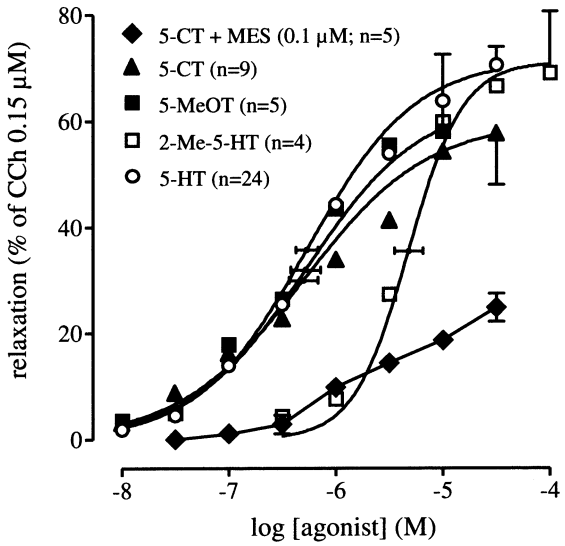

Figure 2 Concentration-relaxation curves to 5-MeOT, 5-CT, 5-HT and 2-Me-5-HT in the human colonic circular muscle preparation in the presence of SB 204070 (10 nM). The curves shown superimposed on the vertically averaged experimental data points represent simulations using the Hill equation and the parameters for upper asymptote (shown with vertical error bars) and midpoint location (shown with horizontal error bars) and the Hill slope that were obtained from the iterative fitting procedure. The data points of the curve to 5-CT obtained in the presence of mesulergine (MES) could not be fitted to the Hill equation and are presented as vertically averaged experimental data points ± s.e.mean.

antagonist granisetron (0.3 μM; $n=6$) failed to affect the curve to 5-HT (ΔpEC_{50} 0.02), thus ruling out involvement of 5-HT_3 receptors (data not shown).

Discussion In the current study, it was demonstrated that in addition to 5-HT_4 receptors, 5-HT_7 receptors also mediate relaxation of human colon circular smooth muscle. First, it was confirmed that 5-HT_4 receptors mediate relaxation (Figure 1) as SB 204070 (10 nM) blocked the relaxation to 5-HT in the presence of methysergide (1 μM). After selective blockade of 5-HT_4 receptors in the absence of methysergide, 5-HT still induced a relaxation. The hypothesis that 5-HT_4 receptors mediated this 5-HT-induced relaxation as a result of insufficient 5-HT_4 receptor blockade by SB 204070 (10 nM), was ruled out by the failure of a 10 fold higher concentration of SB 204070 (100 nM) to further affect the 5-HT-induced relaxation. The inability of tetrodotoxin to affect the 5-HT-induced relaxation suggests that the 5-HT receptor is located on the smooth muscle. Receptors on the smooth muscle that mediate relaxation are most likely coupled to adenylate cyclase activation. Of the known 5-HT receptors, 5-HT_4, 5-HT_6 and 5-HT_7 receptors are positively coupled to adenylate cyclase (Hoyer *et al*., 1994). As SB 204070 (10 nM) adequately blocked 5-HT_4 receptors, 5-HT_6 and 5-HT_7 receptors would be the most likely candidates to mediate the 5-HT-induced relaxation. Involvement of 5-HT_6 receptors, however, was ruled out by the antagonism induced by the potent 5-HT_7 antagonist mesulergine (pK_B 8.3), which possesses only micromolar affinity for 5-HT_6 receptors (Gommeren *et al*., 1998). Mesulergine also binds with nanomolar affinity to 5-HT_2 receptors, but 5-HT_2 receptors are coupled to IP_3 hydrolysis, and, if located on smooth muscle, would be expected to mediate contraction. For the same reason, involvement of 5-HT_1 receptors as well as 5-ht_5 receptors is highly unlikely, as these receptors are negatively coupled to adenylate cyclase, and, if located on the smooth muscle, would not be expected to be associated with relaxation (Hoyer *et al*., 1994).

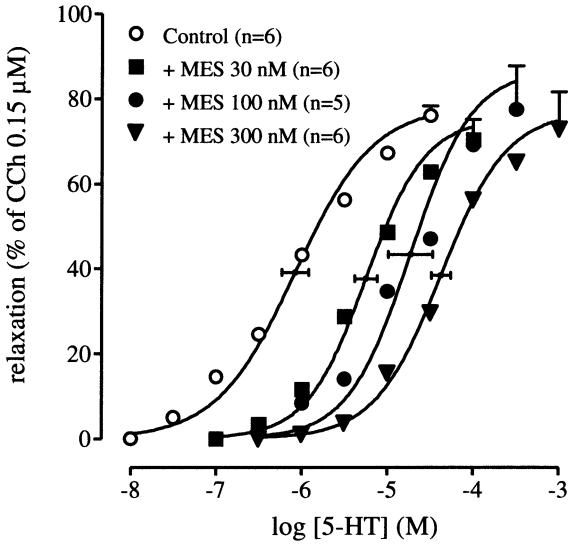

Figure 3 Competitive antagonism by mesulergine (MES) of the 5-HT-induced relaxation of human colonic circular muscle in the presence of SB 204070 (10 nM). The curves shown superimposed on the vertically averaged experimental data points represent simulations using the Hill equation and the parameters for upper asymptote (shown with vertical error bars) and midpoint location (shown with horizontal error bars) and the Hill slope that were obtained from the iterative fitting procedure.

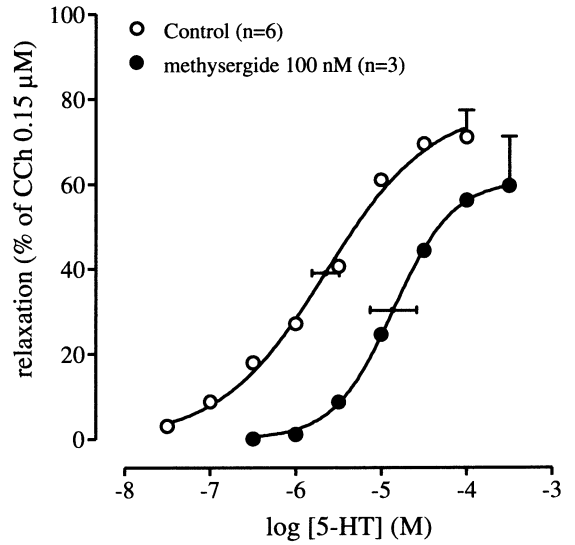

Figure 4 Antagonism by methysergide of the 5-HT-induced relaxation of human colonic circular muscle obtained in the presence of SB 204070 (10 nM). The curves shown superimposed on the vertically averaged experimental data points represent simulations using the Hill equation and the parameters for upper asymptote (shown with vertical error bars) and midpoint location (shown with horizontal error bars) and the Hill slope that were obtained from the iterative fitting procedure.

The pK_B estimate for mesulergine (8.3) against 5-HT agrees well with the affinity estimates obtained in the 5-HT_7 receptor bioassays of the guinea-pig ileum (7.8: Carter *et al*., 1995), rat jejunum (8.1: McLean & Coupar, 1996), and at the cloned human 5-HT_7 receptor expressed in CHO cell lines (8.2: Gommeren *et al*., 1998). It cannot be excluded that the apparent non-monophasic shape of the curve to 5-CT in the presence of mesulergine reflects either insurmountable antagonism by mesulergine or a second relaxant property of 5-CT, in addition to 5-HT_7 receptor agonism. Nevertheless, mesulergine demonstrates that the 5-CT-induced relaxation is primarily 5-HT_7 receptor-mediated. Methysergide is also an antagonist of 5-HT_7 receptors (pA_2 7.6: Carter *et al*., 1995) and the affinity obtained in the current study (pA_2 7.6) points to antagonism of 5-HT_7 receptors. The relaxation to 5-HT, therefore, is likely to be mediated by 5-HT_7 receptors.

Accordingly, the similar potency of 5-CT, 5-HT and 5-MeOT is in good agreement with involvement of 5-HT_7 receptors (Terron, 1998). However, 2-Me-5-HT was markedly less potent, and gave a Hill slope that was twice as steep as the Hill slope of the more potent, equi-efficacious agonists 5-HT, 5-MeOT and 5-CT. The operational model of agonism (Black & Leff, 1983) predicts that when an agonist displays a lower potency accompanied by a markedly steeper Hill slope than other agonists, but the same maximum response, then the agonist does not elicit that response *via* the same receptor or mechanism. Thus, 2-Me-5-HT might not have activated 5-HT_7 receptors in the current study, which is not unlikely, as Zondag *et al*. (1994) reported that 2-Me-5-HT relaxed guinea-pig colonic tissue through activation of α_1-adrenoceptors.

5-HT_7 receptors have been cloned from a number of species including man (for review: see Terron, 1998). Functional correlates were found in several animal species (guinea-pig: Carter *et al*., 1995; cat: Villalon *et al*., 1997; monkey: Leung *et al*., 1996). The first indication that gastrointestinal 5-HT_7 receptors might be present in humans was found by Bard *et al*. (1993), who demonstrated abundant expression of 5-HT_7

receptor mRNA in human brain, colon, ileum and stomach in comparison with low expression in the spleen, liver and kidney. The present study indicates that in the colon, the 5-HT_7 receptor mRNA is translated into functional 5-HT_7 receptors. Additionally, this study might explain why previously, selective 5-HT_4 receptor antagonists (SB 207710 and GR 113808) were found not to behave competitively, as judged from the Schild slopes which were significantly less than unity (McLean & Coupar, 1995; Tam *et al*., 1995). The presence of methysergide and ondansetron (both 10 μM) changed the Schild slope of the selective 5-HT_4 receptor antagonist SB 207710 from 0.5 to 1 (McLean & Coupar, 1995). Thus, methysergide presumably antagonized the 5-HT_7 receptor component (as it did in the present study; Figure 4), exposing a 5-HT-induced relaxation that was entirely mediated by 5-HT_4 receptors.

In conclusion, when 5-HT_4 receptors are blocked, the relaxation of human colon by 5-HT is mediated by smooth muscle 5-HT_7 receptors. The presence of 5-HT_7 receptors might explain the previously reported deviation from simple competitive behaviour of selective 5-HT_4 receptor antagonists when their effects against 5-HT-induced relaxation were assessed. This report provides the first account of functional human (peripheral) 5-HT_7 receptors.

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