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Differences in response to 5-HT_4 receptor agonists and antagonists of the 5-HT_4-like receptor in human colon circular smooth muscle

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1 In isolated circular smooth muscle strips of human colon 5-hydroxytryptamine (5-HT) produced a concentration-related inhibition of spontaneous motility.

2 The azabicycloalkyl benzimidazolones, BIMU 8 and BIMU 1, which have 5-HT_4 receptor stimulant properties, inhibited motility with EC_{50} values of 0.76 μM and 3.19 μM respectively and their E_{max} values were not significantly different from 5-HT (EC_{50}, 0.13 μM).

3 The 5-HT_4 receptor antagonist, DAU 6285 (1-10 μM), displaced the 5-HT concentration-response curve to the right in a parallel concentration-dependent manner without depressing the maximum. The Schild plot was linear and the slope did not differ significantly from unity giving a pA_2 value of 6.32.

4 The high affinity selective 5-HT_4 receptor antagonist, GR 113808, at a concentration of 3 nM displaced the 5-HT concentration-response curve in a parallel manner giving an apparent pK_B estimate of 8.9 $\pm$ 0.24. However, higher concentrations of 10-100 nM GR 113808 did not result in a further significant displacement of the 5-HT concentration-response curve and there was no suppression of E_{max}.

5 GR 113808 (10 nM) also caused a parallel displacement of the concentration-response curve to the 5-HT_4 receptor agonist, 5-methoxytryptamine (5-MeOT) giving apparent pK_B values ranging from 8.3-9.3.

6 GR 113808 (3-100 nM) failed to displace 5-HT or 5-MeOT concentration-response curves in tissue strips from 3 patients out of a total of 10 patients studied in whom the response to 5-HT and 5-MeOT was normal.

7 The 5-HT_4 receptor antagonist, SDZ 205-557 (0.3-10 μM), had no significant effect on 5-HT-induced inhibition of spontaneous motility.

8 The present results are discussed in the light of variability of response to GR 113808 and SDZ 205-557 in other tissues.

9 Overall, our data indicate that human colon circular smooth muscle can be regarded as a site in which 5-HT_4-like receptors are present but it is as yet unclear whether these results are also an indication of receptor variation.

Keywords: DAU 6285; GR 113808; 5-HT_4-like receptors; human colon; SDZ 205-557; benzimidazoles

Introduction

A range of different 5-hydroxytryptamine (5-HT) receptor subtypes have been identified in the gastrointestinal tract. Their stimulation or blockade can result in a variety of motor and secretory changes. The 5-HT_4 receptor subtype which is positively coupled to adenyl cyclase and originally identified by Dumuis *et al.* (1988a,b) has been found to be widely distributed in the gastrointestinal tract of a variety of species. In the guinea-pig ileum (Eglen *et al.*, 1990; Wardle & Sanger, 1993) and colon (Elswood *et al.*, 1991) activation of neuronally located 5-HT_4 receptors induces release of acetylcholine which results in contraction and/or an increase of electrically-evoked contraction. However, in the rat oesophagus (Reeves *et al.*, 1991; Baxter *et al.*, 1991) and ileum (Tuladhar *et al.*, 1991), stimulation of muscle cell located 5-HT_4 receptors results in direct relaxation.

We have previously cited data on the presence of muscle cell-located 5-HT_4-like receptors in the intertaenial circular muscle of human colon, stimulation of which results in relaxation (Tam *et al.*, 1994). Our evidence was based on the rank order of potencies of a range of indole derivatives. Additionally, substituted benzamides which block 5-HT_3 but stimulate 5-HT_4 receptors were agonists on the circular muscle. Tropisetron, a weak 5-HT_4 receptor antagonist but potent 5-HT_3 antagonist (literature pA_2 values approximately 5.8-6.7 and 7.8-10.6 respectively) antagonized the 5-HT and 5-methoxytryptamine (5-MeOT)-induced relaxant responses on the circular muscle with a pK_B value of 6. Ondansetron, methysergide and methiothepin were without effect on 5-HT-induced inhibition of motility. We have now further investigated the 5-HT receptor type on circular muscle of human colon using the azabicycloalkyl benzimidazolones which have improved selectivity of action at 5-HT_4 receptors and the high affinity and selective 5-HT_4 receptor antagonist GR 113808 (Gale *et al.*, 1994). A preliminary account of these studies has been published in abstract form (Hillier *et al.*, 1994).

Methods

Tissues preparation and concentration-response curves

Colon samples from 20 patients undergoing resections for carcinoma were prepared and studied under the conditions described by Tam *et al.* (1994). Twenty per cent of samples were from the ascending colon and the remainder were from

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the descending or sigmoid colon. The region from which we obtained our samples was confirmed histopathologically. Although responses of tissues from different colon regions did not appear to vary, the samples from the ascending colon were small in number. We have not, therefore, rigorously compared regional variations.

In a previous study (Tam *et al.*, 1994) we showed that a cumulative concentration-response curve to 5-HT or 5-MeOT (within the range 0.01–100 μM in final bath concentration) produced concentration-related inhibition of spontaneous activity of the circular muscle of the human colon. A second concentration-response curve repeated 30 min after the first was superimposable upon the first response curves if the spontaneous activity measured immediately before the second constructed response-curve was used as the control for the second curve; the spontaneous activity prior to the second response curve was 51.7 ± 10.7% less than before the first curve ($n = 4$).

Agonist studies

In studies with purported agonists a concentration-response curve to 5-HT was constructed. After washing out and leaving for 30 min a concentration-response curve to BIMU 8, BIMU 1 or DAU 6285 was constructed.

Antagonist studies

Following construction of a concentration-response curve to 5-HT, the 5-HT$_4$ receptor antagonists, DAU 6285 (1–10 μM), GR 113808 (3–100 nM) or SDZ 205-557 (0.3–10 μM) were added for 30 min prior to construction of a second response curve. The level of spontaneous activity after 30 min contact with these antagonists and prior to construction of the second response curve to 5-HT was not different from that observed prior to the second concentration-response curves of control experiments. Only one concentration of antagonist was used in any one strip.

In further studies concentration-response curves were constructed to the 5-HT$_4$ receptor stimulant 5-MeOT. GR 113808 was added at one concentration (10 nM) for 30 min and a second concentration-response curve constructed. *n* values quoted are the number of patients studied.

Data are expressed as mean ± s.e.mean. Statistical analyses were by Student's unpaired *t* test. Equipotent molar ratios (e.p.m.rs) relative to 5-HT were calculated in each tissue examined and expressed as geometric means with 95% confidence limits.

pK_B values for antagonists were calculated using the Schild equation $pK_B = \log_{10}(CR-1) - \log_{10}[B]$ where CR is the concentration ratio of the agonist used in the absence and presence of the antagonist [B] (Arunlakshana & Schild, 1959).

Drugs used

5-Hydroxytryptamine maleate, 5-methoxytryptamine hydrochloride and isoprenaline hydrochloride were purchased from Sigma Chemicals (UK). BIMU 1 ([endo-*N*-8-methyl-8-azabicyclo-(2, 3,1) oct-3-yl]-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazol-1-carboxamide), BIMU 8 ([endo-*N*-8-methyl-8-azabicyclo-2(3,2,1)oct-3-yl]-2,3-dihydro-3-isopropyl-2-oxo-1*H*-benzimidazol-1-carboxamide) and DAU 6285 ([endo-6-methoxy-8-methyl-8-azabicyclo-(3,2,1) oct-3-yl] 2, 3-dihydro-2-oxo-1*H*-benzimidazole-1-carboxylate) were gifts from Dr C.A. Rizzi, Boehringer Ingelheim (Italy). SDZ 205-557 (2-methoxy-4-amino-6-chlorobenzoic acid 2-(diethylamino)ethyl ester) was a gift from Pfizer (U.K.). GR 113808 ([1[2-methylsulphonyl) amino [ethyl] -4-piperidinyl] methyl-1-methyl-1*H*-indole-3-carboxylate) was a gift from Glaxo (UK).

All drugs were dissolved in water at a stock concentration of 10^{-2} M. They were further diluted to the required concentration in Krebs buffer.

Results

Agonist effects

Figure 1 shows that 5-HT, BIMU 8 and BIMU 1 (0.01–100 μM) produced concentration-related inhibitions of spontaneous contractions ($n = 6$). EC_{50} values and E_{max} values for each agonist are shown in Table 1. The rank order of potency was 5-HT > BIMU 8 > BIMU 1. The E_{max} of BIMU 1 and BIMU 8 at 100 μM were not significantly different from that of 5-HT. DAU 6285, another azabicycloalkyl benzimidazolone, however, was relatively inactive as an agonist

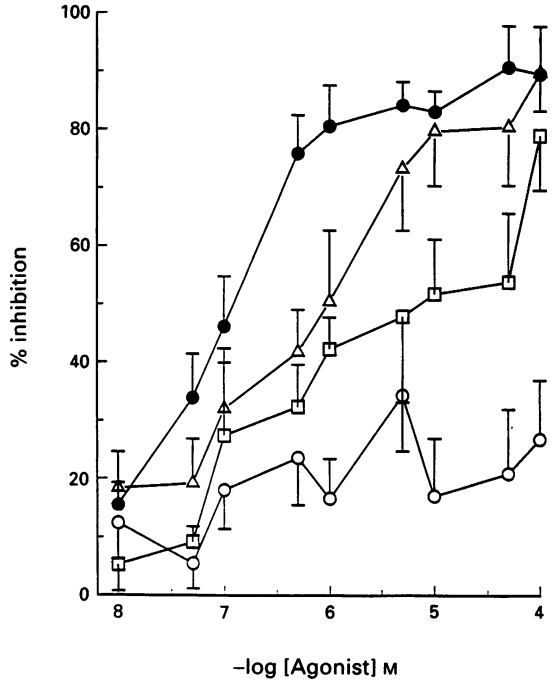

Figure 1 The effect of 5-hydroxytryptamine (5-HT) and some azabicycloalkyl benzimidazolones on the inhibition of spontaneous contractions in the circular muscle of human colon. A cumulative concentration-response curve to 5-HT (●) was constructed and after washing out the drug and leaving for 30 min, a concentration-response curve to BIMU 8 (△), BIMU 1 (□) and DAU 6285 (○) was constructed. Each point is the mean value with s.e.mean. Tissues from 6 patients were utilised in deriving these data. The effect of each benzimidazolone was examined on tissues from each patient.

Table 1 The effect of 5-hydroxytryptamine (5-HT) and azabicycloalkyl benzimidazolones on the spontaneous contractions of the intertaenial circular muscle of human colon

Compound	*EC_{50}* (95% CL)	*e.p.m.r.* (95% CL)	*% maximum inhibition of spontaneous contractions*	n
5-HT	0.13 (0.04–0.42)	1	9.10 ± 7.12	6
BIMU 8	0.76 (0.10–5.91)	4 (0.23–13.5)	90.1 ± 6.61	6
BIMU 1	3.19 (0.32–32.1)	29 (2.63–53.1)	79.2 ± 9.39	6
DAU 6285	–	–	–	6

The EC_{50} value (μM) of each drug is the mean of the EC_{50} values obtained from each patient. The equipotent molar ratios (e.p.m.rs) are the mean ratios calculated from each patient. Other values are mean ± s.e.mean. The number of muscle strips from each patient utilised for each agonist studied was between 1 and 3.
CL, confidence limits; *n*, number of patients.

with concentrations of 0.1–100 μM all causing only approximately 20% inhibition of spontaneous contractions.

Antagonist studies

The 5-HT$_4$ receptor antagonist, DAU 6285 (1, 3, 5 and 10 μM) caused concentration-related parallel displacements in the concentration-response curve to 5-HT while not significantly altering E_{max} (Figure 2a).

The pA_2 value of 6.32 was determined by Schild regression analysis and the slope was 0.84 which was not significantly different from unity (Figure 2b). SDZ 205-557 (0.3–10 μM) was tested as an antagonist in tissues obtained from 13 patients. Overall SDZ 205-557 caused no significant displacements of the concentration-response curves in concentrations up to 10 μM in tissues from 11 patients. The concentration-ratios (95% confidence limits) were 0.3 μM, 0.62 (0.27 – 1.42); 1 μM, 0.62 (0.13 – 2.85); 3 μM, 1.23 (0.51 – 2.96) 10 μM, 1.01 (0.49 – 2.08), $n = 11$. In the tissues from the remaining 2 patients, SDZ 205-557 produced antagonist effects of 5-HT-induced responses with pK_B value of 7.12–8.07, estimated from each concentration of SDZ 205-557. Figure 3 shows the results of the 5-HT$_4$ receptor antagonist, GR 113808 (3–100 nM). GR 113808 showed antagonist effects of 5-HT-induced response in the tissues obtained from 7 patients but was without effect at the same concentrations in 3 patients. In tissues where a response was obtained the antagonism was not, however, concentration-dependent (Figure 3). GR 113808 (3 nM) produced a rightward displacement of the concentration-response curve to 5-HT but at higher concentrations, GR 113808 resulted in little further displacement of the curve to the right. The pK_B values estimated at each concentration are shown in Table 2 and ranged from 7.8–8.9. In tissues from 3 of these 7 patients, the antagonist effect of 10 nM GR 113808 on the agonist 5-MeOT was also studied ($n = 3$). The pK_B values of GR

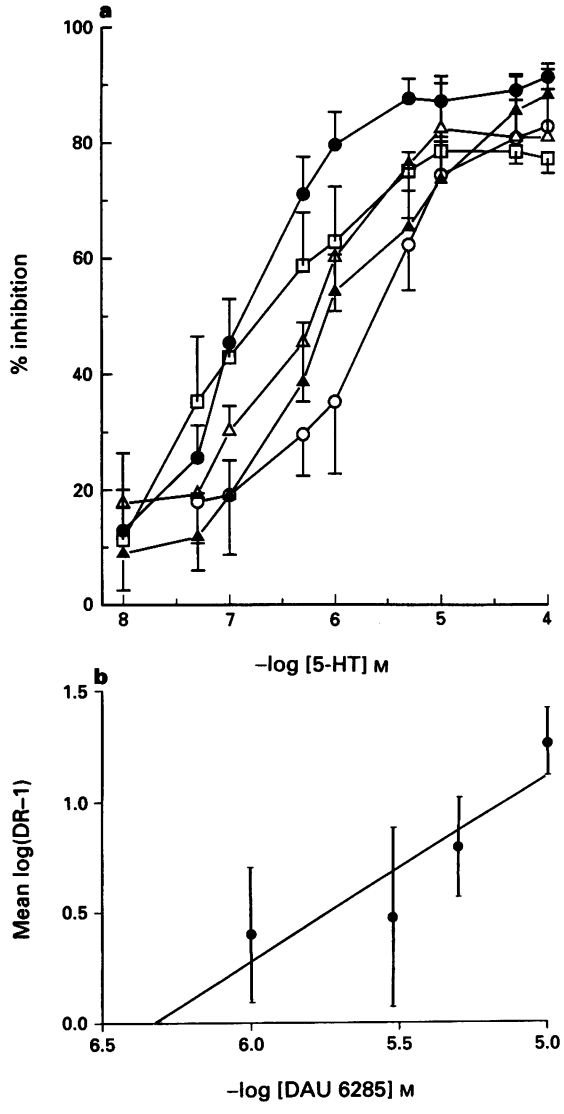

Figure 2 The effect of DAU 6285 on 5-hydroxytryptamine (5-HT)-induced inhibition of spontaneous contractions in the circular muscle of human colon. (a) Cumulative concentration-response curves to 5-HT (0.01–100 μM) were constructed (●). After washing out 5-HT, DAU 6285 at 1 (□), 3 (△), 5 (▲) or 10 (○) μM was added and incubated for 30 min before construction of the second concentration-response curve to 5-HT. The number of patients studied for each concentration of DAU 6285 was 4–6 and 2–3 muscle strips were used from each patient for each concentration-response curve. Other details as in Figure 1. (b) Schild plot of the antagonist effect of DAU 6285 on the 5-HT-induced inhibition of spontaneous contractions of the intertaenial circular muscle of human colon. The pA_2 value of DAU 6285 (6.32) with slope equal to 0.84 (not significantly different from unity). The number of patients studied was 4–6.

Table 2 The effect of GR 113808 on the 5-hydroxytryptamine (5-HT)-induced inhibition of spontaneous contractility in the intertaenial circular muscle of human colon

[GR 113808] (nM)	pK_B (±s.e.mean)	n
3	8.9 ± 0.24	7
10	8.6 ± 0.24	7
30	8.1 ± 0.15	7
100	7.8 ± 0.24	7

The values for each pK_B are derived from the data in Figure 3.

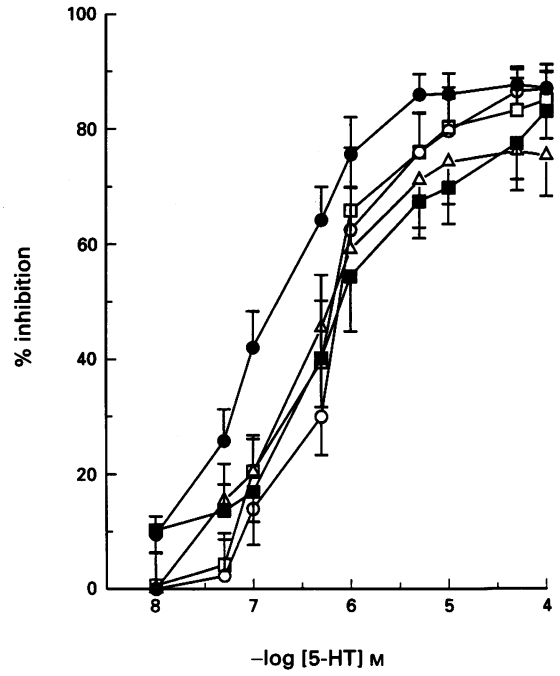

Figure 3 The effect of GR 113808 on 5-hydroxytryptamine (5-HT)-induced inhibition of spontaneous contractions in the intertaenial circular muscle of human colon. Concentration-response curves to 5-HT were constructed cumulatively (0.001–100 nM) with 2 min contact time for each concentration (●). After washing out the 5-HT, GR 113808 at 3 (□), 10 (△), 30 (○) or 100 (■) nM was added and incubated for 30 min before repeating the concentration-response curve to 5-HT. The pK_B values for GR 113808 at each concentration are shown in Table 2. Seven patients were studied and 2–3 muscle strips were utilised from each patient.

113808 (10 nM) against 5-MeOT were as follows with pK_B values against 5-HT in tissues from the same patients shown for comparison in parentheses: 9.3 (7.9); 7.9 (7.9); 8.3 (8.0).

In tissues from 3 of the 10 patients studied in which GR 113808 failed to affect 5-HT, it also failed to inhibit responses to 5-MeOT; the responses to the agonists in these tissues did not, however, differ from the normal response. DAU 6285 (10 μM), SDZ 205-557 (10 μM) and GR 113808 (100 nM) had no significant effect on the concentration-related isoprenaline-induced (0.01–100 μM) inhibition of the spontaneous contractions ($n = 4$, data not shown).

Discussion

Addition of 5-HT to circular muscle strips of human colon results in inhibition of spontaneous contractility. No desensitization to 5-HT was seen under the conditions used. With a range of indoles, substituted benzamides and tropisetron, we showed that the 5-HT receptor on circular muscle fulfilled the pharmacological criteria widely accepted as describing a 5-HT$_4$ subtype. Moreover, the receptors were predominantly located on the smooth muscle cells as tetrodotoxin produced only a small displacement to the right in the 5-HT response curve (Tam *et al.*, 1994); however, we cannot at this time unequivocally exclude the presence of an additional neuronally-located component to the 5-HT response. The response to 5-HT appears to be similar in different regions of the colon but the possibility of regional variation in response requires further appraisal as the majority of the tissues studied were from the descending and sigmoid colon.

In the present study the 5-HT$_4$ receptor-selective benzimidazolone agonists, BIMU 8 and BIMU 1, were found to inhibit spontaneous contractions. The rank orders of potency were similar to those obtained in studies in the mouse embryo colliculi neurones (Dumuis *et al.*, 1991), the rat oesophagus (Baxter & Clarke, 1992) and the guinea-pig ileum (Rizzi *et al.*, 1992; Tonini *et al.*, 1992). The concentration-response curves to BIMU 8 were largely parallel with those of 5-HT and E_{max} was similar. The less active BIMU 1 achieved a similar mean E_{max} but the responses to higher concentrations in this study were more variable. The benzimidazolone, DAU 6285, has been reported to have selective and competitive antagonist actions at 5-HT$_4$ receptors in the mouse embryo colliculi neurone (pK_B = 6.6–6.7, Dumuis *et al.*, 1992), the guinea-pig ileum (pK_B = 6.8–7.0, Waikar *et al.*, 1993) and the rat oesophagus (pK_B = 6.8–7.1, Baxter & Clarke, 1992; Waikar *et al.*, 1993) and the human right atrium (pK_B = 6.8, Schiavone *et al.*, 1991). DAU 6285 also has 5-HT$_3$ receptor antagonist properties (Turconi *et al.*, 1991) but we have shown that the 5-HT$_3$ antagonist, ondansetron, does not affect the 5-HT response curve in the preparation described for this study (Tam *et al.*, 1994). In our hands, DAU 6285 was a competitive inhibitor of the actions of 5-HT at the receptor site in the colon circular muscle causing parallel displacements in the concentration-response curves at concentrations between 1–10 μM. The pA_2 value was 6.3 and the slope of the Schild plot did not differ significantly from unity. In human colon DAU 6285 does appear to have a somewhat lower pK_B value than has been observed in animal studies. This slightly lower value was also found in studies of DAU 6285 on the secretory response in human small intestine (pA_2 = 6.17; Borman & Burleigh, 1993).

GR 113808 is a potent and selective competitive 5-HT$_4$ receptor antagonist in the guinea-pig colon (pK_B = 9.2), rat oesophagus (pK_B = 9.5, Grossman *et al.*, 1993), isolated human right atrium (pK_B 8.8, Kaumann, 1993) and isolated human detrusor muscle (pK_B 8.9, Tonini *et al.*, 1994). In this study 3 nM GR 113808 antagonized the response to 5-HT with an apparent pK_B of 8.9. However, higher concentrations of GR 113808 (up to 100 nM) were not simply competitive and failed to displace the 5-HT response curve significantly further to the right. The E_{max} was not significantly reduced with higher concentrations. The estimated pK_B values using each single concentration of GR 113808 (3–100 nM) ranged from 9.30 to 7.8. A possible explanation is that steady-state conditions may not have been reached in the presence of GR 113808 and 5-HT. However, Gale *et al.* (1994) show that incubation of guinea-pig colon with GR 113808 for periods of 15–60 min produced similar rightward displacements in the concentration-effect curves to 5-HT. GR 113808 was also not simply competitive when it was used to inhibit 5-HT-induced tachycardia in piglet right atrium, an action purported to be via the 5-HT$_4$ receptor. Medhurst & Kaumann (1993) found that 10 nM GR 113808 caused blockade of 5-HT-induced tachycardia but 100 nM caused little further displacement and also reduced the E_{max}. Another 5-HT$_4$ antagonist, SB 203186 (1-piperidinyl)ethyl 1*H*-indole 3 carboxylate) in concentrations of 0.02–10 μM did, however, produce concentration-related displacements in the 5-HT-response curve, with a pK_B of 8.3 and a linear Schild plot, the slope of which did not differ from unity. The authors suggested that GR 113808 may be having a non-specific effect on this system. The proposal that it may also be having a non-specific action on the human colon in inhibiting the response to 5-HT, therefore, requires consideration. This is unlikely, however, as GR 113808 (100 nM) had no significant effect upon isoprenaline-induced relaxation, nor did it suppress E_{max}. Further studies of GR 113808 using more selective 5-HT agonists will help to clarify matters. If with more selective 5-HT$_4$ agonist, GR 113808 does cause concentration-dependent displacements to the right, it would suggest the possibility of the presence of another co-existing receptor subtype contributing to the 5-HT-induced relaxation. We did find that GR 113808 antagonized responses to the 5-HT$_4$ receptor agonist 5-MeOT with pK_B values similar to those seen with 5-HT, but with 5-MeOT it was only possible to use one concentration of GR 113808 (10 nM). An unresolved observation at this time is that in tissues from 3 patients of 10 studied, GR 113808 at 3–100 nM produced no significant antagonism despite the fact that control responses to 5-HT were normal.

SDZ 205-557 has also been utilised as a 5-HT$_4$-selective receptor antagonist which has been shown to block competitively the 5-HT responses at the 5-HT$_4$ receptor in the guinea-pig hippocampus (Eglen *et al.*, 1993), the guinea-pig ileum, (Buchheit *et al.*, 1992), the rat oesophagus (Eglen *et al.*, 1993) and the piglet left atrium (Lorrain *et al.*, 1992) with pK_B's ranging from 7.3–7.5. SDZ 205-557 does seem, however, to vary in its ability to antagonize competitively 5-HT$_4$ receptors. In the piglet left atrium (Lorrain *et al.*, 1992) and in human right atrium (Zerkowski *et al.*, 1993) SDZ 205-557 competitively antagonized 5-HT-induced tachycardia with a pA_2 value of 7.3 and 7.7 respectively. However, in piglet right atrium the inhibitory effect of SDZ 205-557 against 5-HT-induced tachycardias was not simply competitive and the slope of the Schild plot was shallow, making estimates of pK_B values unreliable (Medhurst & Kaumann, 1993). In this study with concentrations of SDZ 205-557 of up to 10 μM we were unable to show any significant displacement of the 5-HT concentration-response curve. In tissues from only 2 patients of the 13 studied was antagonism of 5-HT responses seen with SDZ 205-557.

In conclusion, the 5-HT receptors identified in the intertaenial circular muscle of human colon can be defined as 5-HT$_4$-like receptor type in which azabicycloalkyl benzimidazolones, BIMU 8 and BIMU 1, mimicked the effect of 5-HT. The response to 5-HT can also be antagonized by the potent and selective 5-HT$_4$ receptor antagonists, DAU 6285 and GR 113808 although the effect of the latter is not simply competitive. Further study with other newly-developed selective 5-HT$_4$ antagonists is required to investigate the presence of species and/or tissue differences of this receptor type.

The pharmacologically defined 5-HT$_4$ receptor has now

been cloned and transiently expressed in COS-7 cells. Two splice variants 5-HT$_4$S and 5-HT$_4$L differing in the length and sequence of their carboxy termini have been isolated (Gerald *et al.*, 1994). Regional differences are apparent in that the 5-HT$_4$S transcript is restricted to the rat striatum whereas the 5-HT$_4$L transcript is expressed throughout the brain except in the cerebellum. It is, as yet, uncertain whether this major finding has biological implications for the differences being detected between receptors termed 5-HT$_4$ in human and laboratory animal tissues.

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