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[^{3}H]-Mesulergine labels 5-HT$_7$ sites in rat brain and guinea-pig ileum but not rat jejunum

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1 The primary aim of this investigation was to determine whether binding sites corresponding to the 5-HT$_7$ receptor could be detected in smooth muscle of the rat jejunum. Binding studies in rat brain (whole brain minus cerebellum) and guinea-pig ileal longitudinal muscle were also undertaken in order to compare the binding characteristics of these tissues. Studies were performed using [^{3}H]-mesulergine, as it has a high affinity for 5-HT$_7$ receptors.

2 In the rat brain and guinea-pig ileum, pK_D values for [^{3}H]-mesulergine of 8.0 ± 0.04 and 7.9 ± 0.11 ($n=3$) and B_{max} values of 9.9 ± 0.3 and 21.5 ± 4.9 fmol mg^{-1} protein were obtained respectively, but no binding was detected in the rat jejunum. [^{3}H]-mesulergine binding in the rat brain and guinea-pig ileum was displaced with the agonists 5-carboxamidotryptamine (5-CT) > 5-hydroxytryptamine (5-HT) ≥ 5-methoxytryptamine (5-MeOT) > sumatriptan and the antagonists risperidone ≥ LSD ≥ metergoline > ritanserin > > pindolol.

3 Despite the lack of [^{3}H]-mesulergine binding in the rat jejunum, functional studies undertaken revealed a biphasic contractile response to 5-HT which was partly blocked by ondansetron (1 μM). The residual response was present in over 50% of tissues studied and was found to be inhibited by risperidone > LSD > metergoline > mesulergine = ritanserin > pindolol, but was unaffected by RS 102221 (3 μM), cinanserin (30 nM), yohimbine (0.1 μM) and GR 113808 (1 μM). In addition, the agonist order of potency was 5-CT > 5-HT > 5-MeOT > sumatriptan.

4 In conclusion, binding studies performed with [^{3}H]-mesulergine were able to detect 5-HT$_7$ sites in rat brain and guinea-pig ileum, but not in rat jejunum, where a functional 5-HT$_7$-like receptor was present.

Keywords: 5-HT$_7$ receptor; [^{3}H]-mesulergine; rat jejunum; guinea-pig ileum; rat brain

Introduction

Recent advances in receptor classification using molecular cloning techniques have resulted in the identification of additional 5-HT receptor subtypes such as 5-ht$_5$, 5-ht$_6$ and 5-HT$_7$ to the four major subtypes already present. The 5-HT$_7$ receptor has been cloned from rat (Lovenberg *et al*., 1993; Ruat *et al*., 1993), mouse (Plassat *et al*., 1993), human (Bard *et al*., 1993) and guinea-pig (Tsou *et al*., 1994). Whilst evidence for the endogenous expression and function of the gene products which encode the 5-ht$_5$ and 5-ht$_6$ receptors is yet to be obtained, the 5-HT$_7$ receptor is now recognized as the receptor previously classified as being 5-HT$_1$-like (Tsou *et al*., 1994; Eglen *et al*., 1997).

The 5-HT$_7$ receptor is positively coupled to adenylate cyclase and although there are no selective 5-HT$_7$ receptor ligands available at present, the identification of 5-HT$_7$ sites has been established using the operational criteria of rank order of agonist and antagonist potency at recombinant 5-HT$_7$ receptors (Eglen *et al*., 1994; Sleight *et al*., 1995b). The expressed 5-HT$_7$ receptor has a high affinity (pK_i 8.0–10.0) for 5-carboxamidotryptamine (5-CT), 5-hydroxytryptamine (5-HT), 5-methoxytryptamine (5-MeOT), clozapine, LSD, and mesulergine, moderate affinity (pK_i 6–7.9) for (±)-2-dipropylamino-8-hydroxy-1,2,3,4,-tetrahydronaphthalene (8-OH-DPAT), methysergide, ergotamine and spiperone and low affinity (pK_i < 6.0) for pindolol, cyanopindolol, and buspirone (Hoyer *et al*., 1994; To *et al*., 1995). These attributes comprise a unique pharmacological profile for the 5-HT$_7$ receptor, which distinguishes it from other closely related receptors such as the 5-HT$_{1A}$ receptor.

The 5-HT$_7$ receptor has been identified in regions of rat and guinea-pig brain (Tsou *et al*., 1994; Shenker *et al*., 1987), in particular the limbic system and thalamocortical regions, where it has been suggested that it may have a role in affective behaviours (To *et al*., 1995; Gustafson *et al*., 1996). Functional studies have identified 5-HT$_7$ receptors in porcine vena cava and myometrium (Sumner *et al*., 1989; Kitazawa *et al*., 1998), canine coronary artery (Cushing & Cohen, 1992), marmoset aorta (Dyer *et al*., 1994) and in guinea-pig ileum (Feniuk *et al*., 1984; Kalkman *et al*., 1986; Carter *et al*., 1995).

Until recently, the pharmacological profile of the smooth muscle 5-HT$_7$ receptor was thought to be consistent across species, where activation of the receptor has been shown to result in relaxation. However, smooth muscle contraction by a postjunctional 5-HT$_7$-like receptor in the rat jejunum has also been reported (McLean & Coupar, 1996), raising the possibility of either a different transduction mechanism or further subtypes of the 5-HT$_7$ receptor. The latter is supported by the finding of cDNA in the rat which differed in the carboxy terminal of the seven transmembrane structure of the expressed 5-HT$_7$ receptor (Lovenberg *et al*., 1993; Ruat *et al*., 1993). The generation of receptors differing in the carboxy regions can result in differential coupling to G-proteins (Lucas & Hen, 1995). In addition there have been isolated reports of an apparent species difference in the 5-HT$_7$ receptor. For instance, the affinity of clozapine for the rat 5-HT$_7$ receptor was found to be almost two orders of magnitude greater than the affinity of the compound at the mouse 5-HT$_7$ receptor (Sleight *et al*., 1995b).

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Hence, the primary aim of this investigation was to determine whether binding sites corresponding to 5-HT_7 receptors could be detected in smooth muscle homogenates of rat jejunum. In addition, binding studies in rat brain and guinea-pig ileum were undertaken to identify 5-HT_7 sites, in order to compare the binding characteristics in different tissues. 5-HT_7 sites have been previously labelled in the brain using the agonists [^{3}H]-5-CT and [^{3}H]-5-HT (Sleight *et al*., 1995a; To *et al*., 1995), but often binding with an agonist radioligand may be complicated by the presence of multiple affinity states for the ligand, whereas this is generally not a problem with the use of an antagonist radioligand (Kenakin, 1984).

Methods

Radioligand binding experiments

Membrane preparations Rat brain Hooded Wistar rats of either sex were decapitated followed by rapid removal of the brain. The cerebellum was removed, the remaining mass weighed, and homogenized with 10 volumes of 50 mM Na_2HPO_4 phosphate buffer pH 7.4, using 20 strokes of a glass homogenizer. Following centrifugation at $1000 \times g$ for 10 min at $-2°C$, the supernatant was re-centrifuged at $40{,}000 \times g$ for 20 min at $-2°C$. The resulting pellet was then washed with a further 10 volumes of buffer before the final centrifugation at $40{,}000 \times g$ at $-2°C$ for 20 min. The final pellet was collected and frozen at $-80°C$ until required for radioligand binding studies.

Guinea-pig ileum Homogenates were prepared according to the method of Kalkman *et al*. (1986), who detected a 5-HT_7-like receptor using [^{125}I]-LSD. Briefly, guinea-pigs of either sex were stunned and exsanguinated before removal of the ileum and flushing out of the intra-luminal contents with phosphate buffer. The segments (*ca*. 20 cm) were stretched over a glass rod, to enable separation of the longitudinal and circular muscle layers by gentle rubbing and peeling of the top layer. The wet mass was then collected, weighed, mixed with 10 volumes of buffer, homogenized in an Ultra Turrax set at $0.75 \times$ maximum speed for 10 s, and centrifuged at $700 \times g$ for 10 min at $-2°C$. After centrifugation the buffy coat was skimmed off the surface, and the remaining supernatant and pellet re-homogenized, passed through a double layer of coarse mesh gauze and re-centrifuged at $14{,}000 \times g$ for 30 min. The pellet was collected, then washed with 10 volumes of buffer and re-centrifuged as previously. The final pellet was collected and stored as above.

Rat jejunum Homogenates were prepared according to a similar method employed by Pinkus *et al*. (1990) in an investigation of 5-HT_3 binding sites using [^{3}H]-zacopride. Briefly, hooded Wistar rats of either sex were stunned and exsanguinated before removal of about 20 cm of jejunum, measured from the ligament of Trietz. Phosphate buffer was used to flush out the intra-luminal contents. The intestine was cut vertically and the mucosa scraped off with a glass slide. The remaining tissue was mixed with 50 ml of buffer, homogenized in an Ultra Turrax set at $0.75 \times$ maximum speed for 10 s, and centrifuged at $500 \times g$ for 5 min at $-2°C$. The supernatant was collected and centrifuged at $45{,}000 \times g$ for 20 min. The pellet was then collected, washed with 20 ml of buffer and re-centrifuged as previously. The final pellet was collected and stored as above.

Receptor binding assays

Binding studies were performed using the ligand [^{3}H]-mesulergine. Mesulergine has a high affinity for 5-HT_7 receptors as well as an affinity for 5-HT_{2A} and 5-HT_{2C} receptors, dopamine D_2 receptors and α_1/α_2 adrenoceptors (Closse, 1983; Rinne, 1983; Pazos *et al*., 1985; Hoyer *et al*., 1994). In order to overcome the binding of mesulergine to receptors other than 5-HT_7, masking drugs consisting of cinanserin (30 nM), RS 102221 (3 μM), raclopride (1 μM), prazosin (0.1 μM) and yohimbine (0.1 μM) were used, respectively. The concentrations of the masking drugs were chosen so that there was a theoretical occupancy of at least 90% of their respective receptor with little effect ($<20\%$ in total) on 5-HT_7 receptor binding.

In addition, phosphate buffer (Na_2HPO_4 50 mM) was chosen instead of Tris-HCl (Tris 50 mM, NaCl 140 mM and $MgCl_2$ 5 mM) as it has been reported that the specific binding of [^{3}H]-mesulergine is reduced in the presence of 100 mM or greater of sodium (Closse, 1983). Sodium ascorbate (1 μM) and pargyline (10 μM) were added to the buffer with experiments involving 5-HT.

Saturation curves were constructed in rat brain, jejunum and guinea-pig ileum. In each case, an aliquot of 200 μl containing between 1–1.8, 2–2.5 and 0.8–1.2 mg of protein, respectively, determined by the Bradford method (Bradford, 1976), was used with various concentrations of the radioligand ranging from 0.3–30 nM (final concentration) and buffer containing the masking drugs to give a total volume of 500 μl. Non-specific binding was defined by risperidone (1 μM) and experiments were performed in triplicate.

The assay composition for competition studies in rat brain was as follows: 100 μl of the membrane preparation, 50 μl of the radioligand (10 nM, final concentration), 50 μl of the displacing agent at various concentrations, and buffer containing the masking drugs to give a total volume of 350 μl. Displacing agents were added in 13 different concentrations and the experiment performed in duplicate. In guinea-pig ileum and rat jejunum this was varied slightly to conserve radioligand. The assay medium was as follows: 150 μl of the membrane preparation, 25 μl of the radioligand (10 nM, final concentration), 25 μl of the displacing agent at various concentrations, and buffer containing the masking drugs to give a total volume of 250 μl. Displacing agents were added in at least five different concentrations and the experiment performed in quadruplicate.

In both saturation and competition studies, the drugs and buffer were incubated initially for 10 min at 37°C before addition of the homogenate and incubation at 37°C for a further 60 min. Preliminary studies showed no significant difference in specific binding ($P>0.05$) between experiments incubated for 30, 60 and 90 min. Termination of the experiment was performed by vacuum filtration through Whatman GF/B filters presoaked in 0.5% polyethylene glycol and risperidone (1 μM). Each filter was placed in a plastic vial with 5 ml of Filtercount (Packard), vortexed, and the radioactivity determined by a scintillation counter (Packard Tricarb 2000, CA, U.S.A.) after at least 5 h had elapsed.

Functional studies

Hooded Wistar rats of either sex were stunned and exsanguinated before removal of about 20 cm of jejunum, measured from the ligament of Trietz (McLean & Coupar, 1996). The intraluminal contents were flushed out using warm modified Krebs-Henseleit solution of the following composi-

tion (mM): NaCl 116, KCl 5.4, $MgSO_4$ 0.6, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1. Four segments of jejunum (3–4 cm in length), were set up separately on tissue holders under 1 g weight tension and equilibrated in 20 ml organ baths containing Krebs-Henseleit solution gassed with 95% O_2: 5% CO_2 and maintained at 37°C. Responses were measured using an isotonic transducer (HUgo Basile) connected to a Grass model 79D polygraph recorder. Following equilibration, non-cumulative concentration-response curves were constructed for the agonists to avoid desensitization, as reported by McLean & Coupar (1996). Preliminary experiments showed non-reproducible curves, in agreement with previous findings in the rat jejunum (McLean & Coupar, 1996), therefore only one concentration-response curve was recorded in each tissue segment. Curves were established as described by addition of increasing concentrations of the agonist to the bathing solution at 10 min intervals, and agonists left in contact with the tissues for < 30 s. Paired segments were used in order to measure the effect of antagonists, one always serving as control and the others to compare the response of the agonist in the presence of different antagonist concentrations. The effect of the agonists in the absence and presence of antagonist were expressed as a percentage of a maximum contraction obtained with acetylcholine (1 μM) in each segment at the end of the experiment. Antagonists were incubated for 40 min before testing of agonists.

Drugs

The following drugs were used: 5-carboxamidotryptamine, cinanserin, lysergic acid diethylamide (LSD), metergoline, 5-methoxytryptamine, (−) pindolol, raclopride L-tartrate, risperidone, tetrodotoxin citrate (Research Biochemicals International, Natick, MA, U.S.A.), acetylcholine chloride, atropine hydrochloride, 5-hydroxytryptamine creatinine sulphate, pargyline hydrochloride (Sigma, Poole, U.K.), prazosin hydrochloride, yohimbine hydrochloride (ICN Biomedicals, Aurora, OH, U.S.A.), haloperidol (Searle, Melbourne, Australia), [^{3}H]-mesulergine (Amersham, Amersham, U.K.), RS 102221 8-[5-(2,4-dimethoxy-5-(4-trifluromethylphenylsulphonamido)phenyl-5-oxopentyl]-1,3,8 triazaspiro[4.5]decane-2,4-dione (Tocris Cookson, Bristol, U.K.), ritanserin (Janssen-Cilag, Sydney, Australia), GR 113808 ({1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl}methyl 1-methyl-1H-indole-3-carboxylate, ondansetron hydrochloride, sumatriptan succinate (Glaxo, Melbourne, Australia). Ritanserin, pindolol and cinanserin were dissolved in methanol, ondansetron, risperidone and metergoline were dissolved in ethanol and RS 102221 was dissolved in dimethylsulphoxide (DMSO) before dilution to the required concentrations. The volumes of solvent used constituted less than 0.02% of the final concentration in both functional and binding studies and had no effect in either study. All other drugs were dissolved in distilled water.

Data analysis

Equilibrium dissociation constants (K_D) for saturation studies were obtained using non-linear regression analysis and competition data were analysed according to one or two-site binding models. The equation used for fitting one site competition curves was:

$$y = \min + (\max - \min)/(1 + 10^{\times - \mathrm{Log\,IC_{50}}}),$$

where min is the apparent minimum, max is the apparent maximum and Log IC_{50} is the logarithm of the concentration of the competing drug, ×, required to inhibit the binding of the radioligand by 50%. The equation used for fitting two-site competition curves was:

$$y = \min + (\max - \min)[F1/(1 + 10^{\times - \mathrm{Log\,IC_{50(1)}}}) + (1 - F1)/(1 + 10^{\times - \mathrm{Log\,IC_{50(2)}}})],$$

where min is the apparent minimum, max is the apparent maximum, F1 is the fraction of receptors with an affinity described by Log $IC_{50(1)}$ (the logarithm of the concentration of the competing drug, ×, required to inhibit the binding of the radioligand by 50%) and the remaining receptors have an affinity described by Log $IC_{50(2)}$. K_i values were obtained using the Cheng & Prusoff equation (1973).

Contractions to serotonergic agonists were expressed as a percentage of the maximum contraction obtained to acetylcholine (1 μM). The EC_{50} values, concentrations producing 50% of the maximum response elicited by the agonists, were estimated from non-linear regression plots of single curves. The data was analysed by fitting a logistic curve of the form:

$$y = \min + \textstyle\sum_{i=1,2,3\ldots n} (max_i - min)/(1 + 10^{(Log\,EC_{50i} - \times)^{nHi}}),$$

where i is apparent number of curves, max is the apparent maximum, min is the minimum point, Log EC_{50} is the logarithm of the concentration, ×, producing 50% of the maximum response of each individual curve and n_H is the slope factor. Antagonist potencies were expressed as either a pK_B or an apparent pK_B value. Initially, pA_2 values were calculated from experiments in which three concentrations of antagonist were tested, using the method of Arunlakshana & Schild (1959). The slope of the Schild plot was calculated and compared to a slope of unity as expected for a competitive antagonist, using Student's unpaired *t*-test. When the slope did not differ significantly from unity ($P > 0.05$), an estimate of the pK_B value was made by fitting a regression with the slope constrained to unity. Apparent pK_B values were calculated from the relationship:

$$pK_B = \log(CR - 1) - \log[B],$$

where CR is the concentration ratio of the agonist in the presence and absence of antagonist (B), (Furchgott, 1972).

Student's *t*-test was used for comparison of individual means and Dunnett's test was used when multiple means were compared to a common control; the criterion for statistical significance for both tests was set at $P < 0.05$. Arithmetic and geometric means are given with mean ± s.e.mean or mean with 95% confidence intervals, respectively. Pearson correlation coefficients were calculated using the computer program Graph Pad Prism 2.0 (GraphPad Software, San Diego, CA, U.S.A.). All other calculations and graphics were also performed using Graph Pad Prism 2.0.

Results

Saturation curves in the rat brain were performed in the presence of raclopride (1 μM) to inhibit the binding of [^{3}H]-mesulergine to dopamine D_2 receptors. The additional inhibition of α-adrenoceptors with prazosin (0.1 μM) and yohimbine (0.1 μM) and 5-HT_{2A} receptors using cinanserin (30 nM) had little effect on the specific binding of the radioligand. However, inhibition of 5-HT_{2C} receptors using RS 102221 was found to significantly ($P < 0.05$) decrease the specific binding of [^{3}H]-mesulergine (0.3–30 nM), indicating the possibility of non-homogeneous binding sites recognized by the radioligand (Figure 1).

At a concentration of 0.3 μM (100 × K_D value at 5-HT_{2C} receptors), RS 102221 did not sufficiently block 5-HT_{2C} receptors when 30 nM of [^{3}H]-mesulergine was used. This is

illustrated in Figure 1, where an increase in specific binding occurred when 30 nM of [^{3}H]-mesulergine was used. To overcome this problem, the concentration of RS 102221 was increased 10 fold to 3.0 μM. This concentration was found to be sufficient in reducing the counts obtained when using 30 nM of [^{3}H]-mesulergine, and the curve reached a plateau as previously. A pK_D value of 8.0 $\pm$ 0.04 and a B_{max} of 9.9 $\pm$ 0.3 fmol mg protein^{-1} (n = 3) was calculated.

The rank order of potency of the displacing drugs studied in the rat brain was as follows: (agonists) 5-CT > 5-HT $\geqslant$ 5-MeOT > sumatriptan and (antagonists) risperidone $\geqslant$ LSD $\geqslant$ metergoline > ritanserin > pindolol (Table 1). All agonists best fitted a two-site competition model (Figure 2a, Table 1) and all antagonist ligands best fitted a one-site competition model except for ritanserin which best fitted a two-site model (Figure 2b, Table 1). In a preliminary experiment, haloperidol (0.01 nM to 1 μM), a compound with weak affinity at 5-HT$_7$ receptors, did not displace [^{3}H]-mesulergine binding. The correlation between the pK_i values obtained in the rat brain and previously published values from recombinant 5-HT$_7$

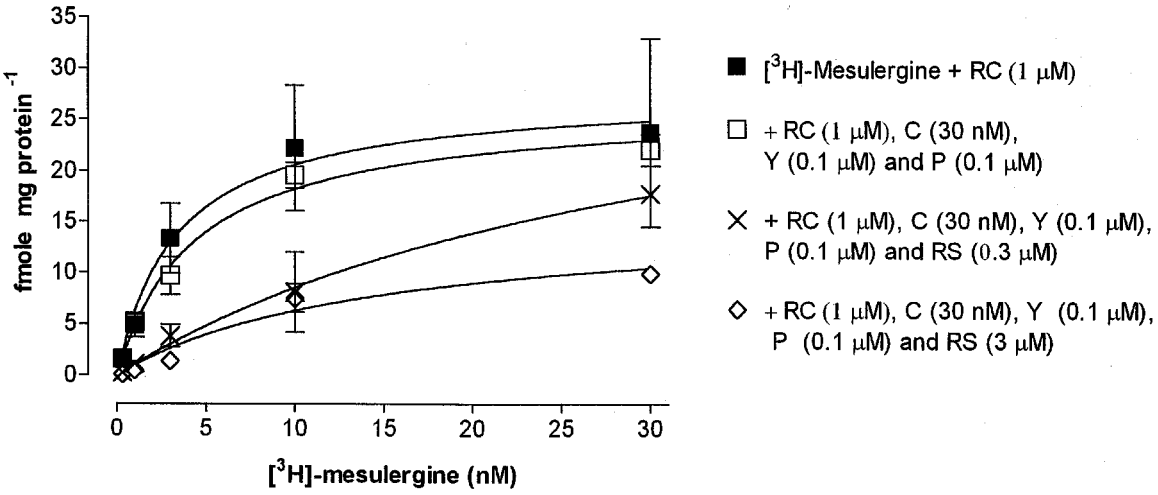

Figure 1 Saturation curve of [^{3}H]-mesulergine binding in the rat brain in the absence and presence of yohimbine (Y: 0.1 μM), prazosin (P: 0.1 μM) cinanserin (C: 30 nM) and RS 102221 (RS: 0.3 and 3 μM). All experiments were performed in the presence of raclopride (RC: 1 μM). Abscissa: Concentration of [^{3}H]-mesulergine in nM; Ordinate: fmole mg of protein^{-1}. Each point is the mean $\pm$ s.e.mean from three experiments.

Table 1 Comparison of ligand affinities in guinea-pig ileum, rat brain and isolated rat jejunum. All data are expressed as mean values with 95% confidence intervals noted in parentheses. High and low affinity values as well as the per cent fraction are shown for compounds displaying two-site competition

Compound	*Rat brain* pK_i *or* pK_D	*Guinea-pig ileum* pK_i *or* pK_D	*Rat jejunum* pK_B *or apparent* pK_B
Agonists			
5-CT	11.4 (11.0–11.7) 39% 8.0 (7.7–8.3) 61% n=3	12.1 (11.6–12.6) 46% 9.4 (9.0–9.9) 54% n=4	–
5-HT	9.9 (9.5–10.3) 35% 7.2 (6.8–7.0) 65% n=3	10.0 (9.3–10.7) 47% 8.8 (8.1–9.5) 53% n=3	–
5-MeOT	9.2 (8.8–9.6) 43% 6.2 (5.9–6.5) 57% n=3	9.6 (8.8–10.4) 32% 7.7 (7.2–8.2) 68% n=3	–
Sumatriptan	7.6 (7.2–8.1) 30% 5.0 (4.8–5.2) 70% n=3	7.0 (6.5–7.6) 46% 5.1 (4.5–5.6) 54% n=3	–
Antagonists			
Risperidone	8.3 (7.7–8.9) n=4	9.4 (9.0–9.8) n=3	8.9 (8.4–9.4)* n=3
LSD	8.1 (8.0–8.3) n=4	9.4 (8.8–10.7) n=3	7.6 (7.3–7.9)* n=3
Metergoline	8.0 (7.9–8.1) n=4	8.8 (8.5–9.0) n=3	7.8 (7.5–8.1) n=3
Mesulergine	8.0 (7.9–8.2)# n=3	7.9 (7.6–8.2)# n=3	7.3 (7.2–7.4) n=3
Ritanserin	7.6 (6.9–8.3) 51% 5.7 (5.2–6.2) 49% n=4	8.3 (8.1–8.5) n=3	7.3 (8.3–6.3)* n=3
Pindolol	4.5 (4.3–4.7) n=4	6.3 (5.8–6.4) n=3	6.7 (6.0–7.3)* n=3

*Apparent pK_B value; # pK_D value.

receptors obtained from rat or mouse and expressed in transfected cells was significant ($P<0.05$); a Pearson correlation factor of 0.90 was obtained (Figure 3).

Saturation studies in the guinea-pig ileum were performed in the presence of the concentrations of masking drugs found to be optimum for 5-HT_7 receptor binding in the rat brain, under these conditions (see Methods). The pK_D value of [^{3}H]-mesulergine was calculated to be 7.9 ± 0.11 and the B_{max} was 21.5 ± 4.9 fmol mg protein^{-1} ($n=3$).

The order of affinity of the agonist and antagonist displacers studied was the same as in the rat brain with some slightly higher pK_i values, see Table 1. All agonist ligands best fitted a two-site model whilst all antagonist ligands best fitted a one-site competition model (Figure 2c and d). The correlation between the pK_i values obtained in the guinea-pig ileum and recombinant rat or mouse 5-HT_7 receptors was significant ($P<0.05$); a Pearson correlation factor of 0.94 was obtained (Figure 3).

Similar binding studies to those performed in the rat brain and guinea-pig ileum using [^{3}H]-mesulergine were undertaken in the rat jejunum, but no binding to the 5-HT_7 receptor was detected.

Effect of 5-HT in the absence and presence of ondansetron in the isolated rat jejunum

In agreement with the findings of McLean & Coupar (1996) a biphasic concentration-response curve for 5-HT (10 nM–3 μM) induced contractions were obtained in the rat jejunum (Figure 4). A biphasic model better described the interaction of 5-HT in the rat jejunum than a monophasic model with R^2 values of 0.83 and 0.79 respectively. The pEC_{50} value of the first phase of the curve was 7.58 ± 0.12 with a maximum of 34.1 ± 4.4% of the response to acetylcholine (1 μM). The pEC_{50}

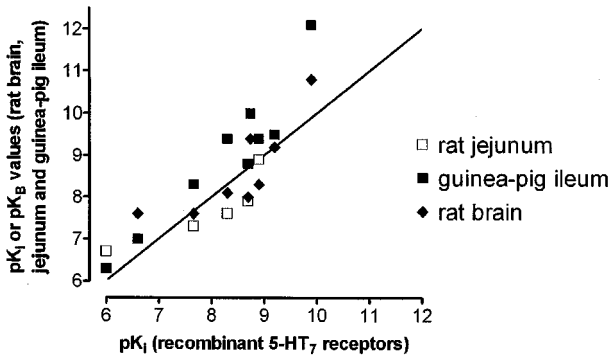

Figure 3 Correlation between pK_i values obtained in recombinant 5-HT_7 receptors from rat or mouse and expressed in transfected cells (Roth *et al*., 1994; Plassat *et al*., 1993; Ruat *et al*., 1993; Shen *et al*., 1993) and pK_i or pK_B values obtained in rat brain, guinea-pig ileum and rat jejunum. Abscissa: pK_i values for recombinant 5-HT_7 receptors, Ordinate: pK_i or pK_B values obtained in rat brain and jejunum and guinea-pig ileum. The continuous line represents a line of identity.

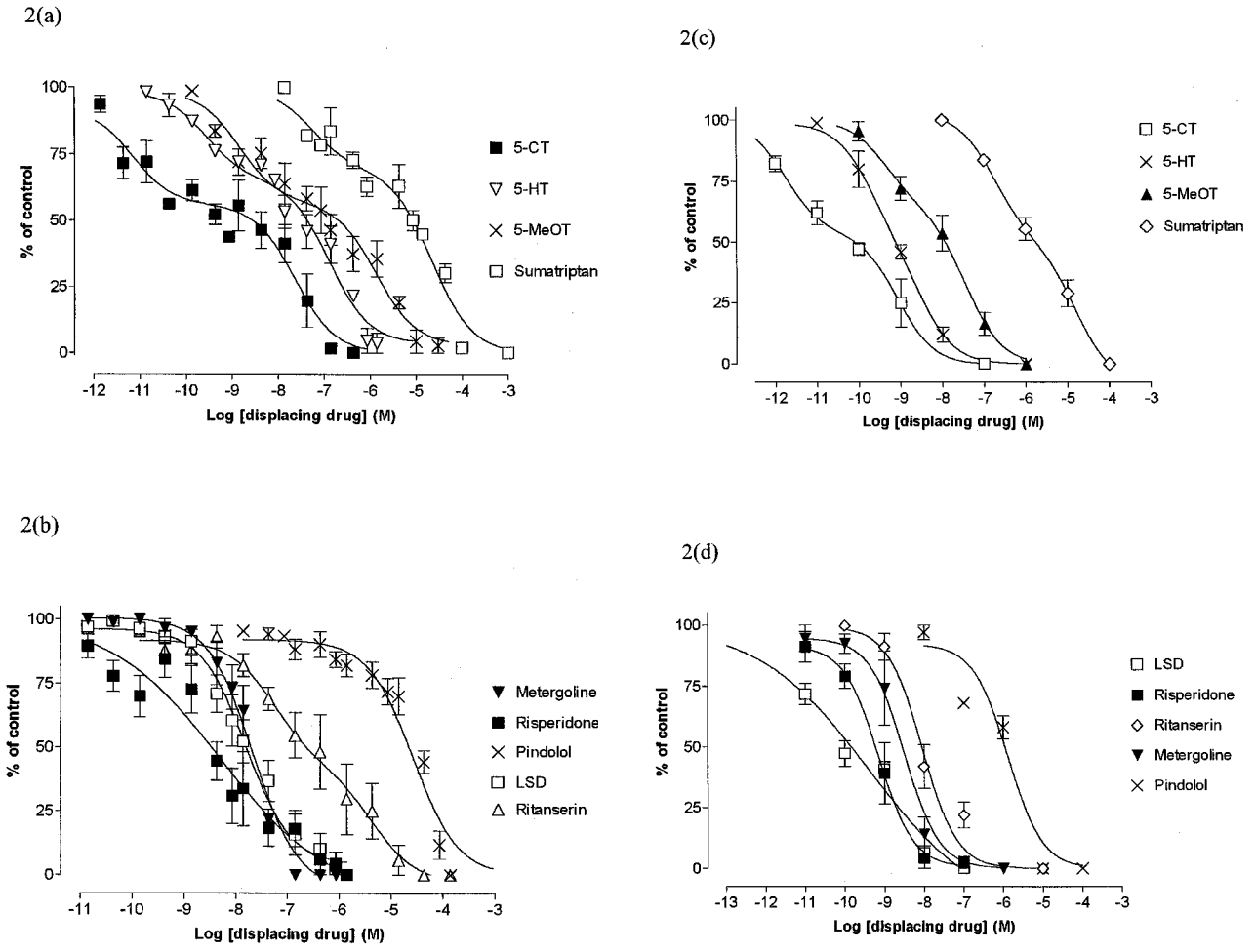

Figure 2 (a, b) inhibition of [^{3}H]-mesulergine binding to rat brain and (c, d) guinea-pig ileum by various ligands. Abscissa: Log molar concentration of the displacing drug, Ordinate: % of specific binding. The data represent the mean$\pm$s.e.mean per cent of maximum specific binding (defined with 1 μM risperidone) of at least three experiments.

value of the second phase of the curve was 6.54±0.17 with a maximum of 69.5±8.5 of the response to acetylcholine (1 μM). However, in the presence of ondansetron (0.1, 1 and 10 μM) the curve flattened and was better defined by a monophasic curve, (pEC_{50} = 6.79±0.07). There was no significant difference ($P>0.05$) between the curves obtained in the presence of 0.1, 1 and 10 μM of ondansetron. In each case ondansetron significantly reduced the maximum response of 5-HT by *ca.* 32% ($P<0.05$, $n=4$). Thus ondansetron (1 μM) was included in all further experiments involving 5-HT in order to investigate the non-5-HT_3 receptor-mediated response. However, a response to 5-HT in the rat jejunum after blockade of 5-HT_3 receptors with ondansetron was not always present, *ca.* 50% of tissues failed to respond to the agonist, even though a response to acetylcholine was present.

Effect of 5-CT, 5-MeOT and sumatriptan

5-CT (3 nM – 3 μM) resulted in a concentration-response curve similar to the monophasic curve of 5-HT. The pEC_{50} of 5-CT (7.03±0.07) was significantly higher ($P<0.05$) than that of 5-HT (6.79±0.07) in the presence of ondansetron (1 μM), however the maximum contraction produced (37.1±2.7% of the contraction to Ach, 1 μM) was not significantly different to that produced by 5-HT (36.9±1.8%).

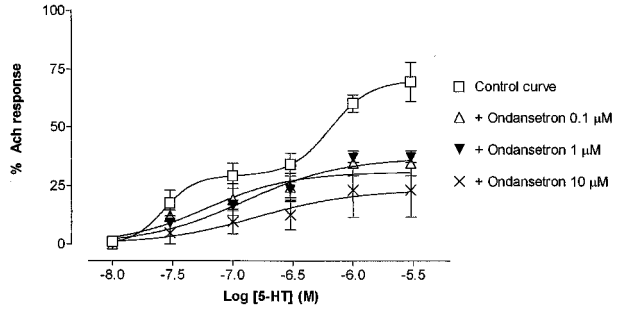

Figure 4 Concentration-response curve for the contractile effect of 5-HT in the absence and presence of ondansetron (0.1, 1 and 10 μM) in isolated rat jejunum. Abscissa: Log molar concentration of 5-HT, Ordinate: % of maximum contraction obtained with acetylcholine (1 μM). The data represent the mean±s.e.mean of four paired experiments.

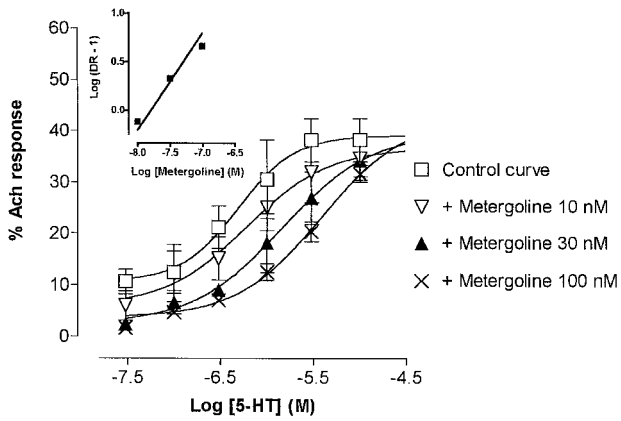

Figure 5 Concentration-response curves for 5-HT in the absence and presence of metergoline (10, 30 and 100 nM). Abscissa: Log molar concentration of 5-HT, Ordinate: % of maximum contraction obtained with acetylcholine (1 μM). The data represent the mean± s.e.mean of three paired experiments. All experiments were conducted in the presence of ondansetron (1 μM). The slope of the Schild regression (inset) was 0.8±0.09.

The pEC_{50} of 5-MeOT (5.33±0.11) was significantly lower ($P<0.05$) than that of 5-HT (6.79±0.07) in the presence of ondansetron (1 μM), however the maximum contraction produced (46.7±7.2% of the contraction to Ach, 1 μM) was greater but not significantly different to that produced by 5-HT ($P>0.05$).

A pEC_{50} value of <5 was obtained for sumatriptan, with a maximum contraction of only 11.3±3.1% of the contraction to Ach, (1 μM).

Effect of 5-HT in the absence and presence of various antagonists

The effect of 5-HT in the presence of risperidone (10–100 nM), ritanserin (100 nM – 1 μM), LSD (10–100 nM), metergoline (10–100 nM), mesulergine (10–100 nM) and pindolol (1 and 3 μM) was investigated. At a concentration of 10 nM, risperidone and ritanserin caused both a shift in the control curve to 5-HT and a depression of the maximum response to the agonist. However, at higher concentrations of the antagonists, neither the depression of the maximum nor the shift in the control curve was increased. LSD also resulted in a depression of the maximum response with no further shift of the 5-HT curve when used at 30 nM, and this effect was increased at a concentration of 100 nM of LSD. Thus apparent pK_B values were reported for risperidone, ritanserin and LSD (Table 1). Schild slopes of 1.0±0.04 (data points; 9) and 0.8±0.09 (9) were obtained for mesulergine and metergoline (Figure 5) respectively. These values were not significantly different ($P<0.05$) from unity. The correlation between the K_B and apparent pK_B values obtained in the rat jejunum and recombinant 5-HT_7 receptors from rat or mouse expressed in transfected cells (Hoyer *et al.*, 1994) was significant ($P<0.05$); a Pearson correlation factor of 0.87 was obtained (Figure 3).

Preliminary studies showed RS 102221 (3 μM), yohimbine (0.1 μM), cinanserin (30 nM) and GR 113808 (1 μM) to have no effect on the contractile response to 5-HT in the rat jejunum, with pEC_{50} values of 5-HT in the presence of the antagonists not significantly different to that of the control curve, ($P>0.05$). Atropine (1 μM) and tetrodotoxin (1 μM) failed to significantly modify ($P>0.05$) the non 5-HT_3-mediated response.

Discussion

The results from the present study suggest the presence of 5-HT_7 receptor binding sites in rat brain and guinea-pig ileum, but not in rat jejunum where a functional 5-HT_7-like receptor was detected.

Receptor characterization

Due to the lack of selective and specific ligands currently available for studying the 5-HT_7 receptor, the use of rank order of agonist and antagonist affinity was used to characterize the 5-HT_7 site, as has been undertaken previously in studies using expressed and native 5-HT_7 receptors (Kalkman *et al.*, 1986; Plassat *et al.*, 1993; Monsma *et al.*, 1993; Erlander *et al.*, 1993; To *et al.*, 1995; Carter *et al.*, 1995; McLean & Coupar, 1996).

The presently described results obtained in rat brain are in agreement with findings in both guinea-pig and rat brain of 5-HT_7 sites, radiolabelled using serotonin receptor agonists (Sleight *et al.*, 1995a; To *et al.*, 1995; Gustafson *et al.*, 1996).

A number of masking drugs were used in the present study in order to inhibit the binding of [^{3}H]-mesulergine to other receptors for which it has affinity. Mesulergine has been reported to have affinity for 5-HT_{2A} and 5-HT_{2C} receptors as well as some affinity for dopamine D_2 and α_1/α_2-adrenoceptors (Closse, 1983; Rinne, 1983; Hoyer *et al*., 1994), (see Methods). The masking drugs, some of which also have affinity for 5-HT_7 receptors, were chosen at concentrations which would theoretically occupy at least 90% of their targetted receptor populations (Kalkman *et al*., 1986; Hoyer *et al*., 1994; Sleight *et al*., 1995a; Bonhaus *et al*., 1997), without significantly affecting the binding of the radioligand to the 5-HT_7 receptor.

Saturation studies performed in rat brain and guinea-pig ileum provided pK_D values of 8.0 ± 0.04 and 7.9 ± 0.11 respectively for [^{3}H]-mesulergine, which were in accordance with a previously reported pK_B value of 7.8 (Carter *et al*., 1995) and a pK_i value of 8.15 (Hoyer *et al*., 1994; To *et al*., 1995) at the 5-HT_7 receptor. However, no evidence of specific binding to a 5-HT_7 site was apparent in the rat jejunum.

Displacement of [^{3}H]-mesulergine binding in rat brain and guinea-pig ileum was observed using the agonists 5-CT (pK_i = 11.4, 12.1), 5-HT (9.9, 10.0), 5-MeOT (9.2, 9.6) as well as sumatriptan (7.6, 7.0) and the antagonists risperidone (8.3, 9.4), ritanserin (7.6, 8.3), metergoline (8.0, 8.8), LSD (8.1, 9.4) and pindolol (4.5, 6.3), respectively. The pK_i values of these compounds correlated well with those from other studies of the 5-HT_7 receptor in both order and magnitude (Kalkman *et al*., 1986; Bard *et al*., 1993; Plassat *et al*., 1993; Ruat *et al*., 1993; Shen *et al*., 1993; Roth *et al*., 1994; To *et al*., 1995).

5-CT, 5-HT, 5-MeOT and sumatriptan displayed two-site displacement binding in the rat brain and guinea-pig ileum, which is not unusual for agonists, since they can bind to both a high and low affinity state of the receptor (Kenakin, 1984). However, the two-site binding displayed by ritanserin in the rat brain is more difficult to explain, and was not detected in the guinea-pig ileum nor reported in guinea-pig brain using [^{3}H]-5-CT (To *et al*., 1995).

The possibility of [^{3}H]-mesulergine binding to non-serotonergic receptors, such as dopamine receptors, were ruled out by the addition of masking drugs such as raclopride (Rinne, 1983) and by the use of the agonists 5-CT, 5-HT and 5-MeOT which are specific for serotonin receptors. In addition, a preliminary experiment performed with haloperidol (0.01 nM to 1 μM), a potent D_2 receptor antagonist (pK_i = 9.3), with little affinity for 5-HT_7 receptors (pK_i = 6.6; Roth *et al*., 1994), failed to displace mesulergine binding in rat brain.

The binding site identified in both rat brain and guinea-pig ileum is unlikely to be of the 5-HT_{1A} or 5-HT_{1B} (rat brain) subtype, since pindolol was found to have a low (micromolar) affinity in this study, consistent with 5-HT_7 but not 5-HT_1 receptors, where it has nanomolar affinity (Lovenberg *et al*., 1993; Ruat *et al*., 1993; Hoyer *et al*., 1994; Carter *et al*., 1995; McLean & Coupar, 1996). The other 5-HT_1 receptor subtypes; 5-HT_{1D} (guinea-pig ileum), 5-ht_{1E}, and 5-ht_{1F} were ruled out by the high affinity of 5-CT in both the rat brain (pK_i = 11.4) and guinea-pig ileum (pK_i = 12.1, Hoyer *et al*., 1994).

The receptor is also unlikely to be the 5-HT_{2A} or 5-HT_{2C} subtype, firstly because these receptors were excluded with cinanserin and RS 102221, respectively, and more importantly the antagonist order of affinity of risperidone (pK_i = 8.7, 9.4), metergoline (8.0, 8.8) and ritanserin (7.7, 8.3) in rat brain and guinea-pig ileum, respectively did not correlate with either 5-HT_{2A} (risperidone > ritanserin > metergoline) or 5-HT_{2C} (metergoline > ritanserin > risperidone) receptors (Hoyer *et al*., 1994, Sleight *et al*., 1995a). In addition, 5-CT was found to have nanomolar affinity in this study whilst it has micromolar affinity or lower at both 5-HT_{2A} and 5-HT_{2C} receptor sites (Bard *et al*., 1993; Hoyer *et al*., 1994; Roth *et al*., 1994). Furthermore, sumatriptan was found to have micromolar affinity in this study whilst it has only millimolar affinity at 5-HT_{2A} and 5-HT_{2C} receptors (Hoyer *et al*., 1994).

The high affinity of 5-CT and 5-MeOT also ruled out the involvement of 5-HT_3 receptors, where the agonists are inactive (Bard *et al*., 1993, Hoyer *et al*., 1994 Sleight *et al*., 1995b).

The involvement of 5-HT_4, 5-ht_5 and 5-ht_6 receptors is excluded due to the use of a nanomolar concentration of [^{3}H]-mesulergine in displacement studies. Mesulergine has only micromolar affinity at 5-ht_{5A}, 5-ht_{5B} and 5-ht_6 receptors (Plassat *et al*., 1993; Erlander *et al*., 1993; Monsma *et al*., 1993) and is inactive at 5-HT_4 receptors (Bard *et al*., 1993). Further evidence against 5-ht_{5B} and 5-ht_6 receptor involvement is the low affinity of 5-CT (pK_i = 7.4 and 6.6 respectively) for these receptors (Hoyer *et al*., 1994).

While the 5-HT orphan receptor reported by Castro *et al*. (1997) has a high affinity for 5-CT, it cannot be the receptor in question since mesulergine has only a micromolar affinity at the orphan receptor (Castro *et al*., 1997). In addition the 5-HT orphan receptor has a similar affinity for both 5-CT and 5-HT (Castro *et al*., 1997), a characteristic not displayed by the receptor in the present study.

Functional significance

5-HT_7 sites have been found to exist in high densities in the limbic system, hypothalamus and thalamocortical regions, hence leading to the suggestion that they may have a role in affective behaviours (Plassat *et al*., 1993; Eglen *et al*., 1994; Gustafson *et al*., 1996).

This suggestion is further supported by the high affinity of atypical antipsychotics such as clozapine and risperidone for the 5-HT_7 receptor (Roth *et al*., 1994). Moreover, the 5-HT_7 receptor has also been implicated in other affective disorders such as depression, where it was found that a down-regulation of the receptor occurs after chronic antidepressant treatment (Sleight *et al*., 1995a). In addition 5-HT_7 receptors may have a role in the regulation of mammalian circadian rhythms (Lovenberg *et al*., 1993).

The high affinity binding of LSD in rat brain in the present study adds further support to a 5-HT_7 site where a pK_i value similar to that reported in guinea-pig brain by To *et al*. (1995) was obtained (pK_i 8.2 compared with 7.8). This raises the possibility that the hallucinogenic action of LSD may be mediated in part *via* the 5-HT_7 receptor.

Peripheral tissue studies

The results of the binding studies in guinea-pig ileum lend further support to the functional findings of Feniuk *et al*. (1984), Kalkman *et al*. (1986) and Carter *et al*. (1995) where a postjunctional 5-HT site was seen to induce relaxation of pre-contracted guinea-pig ileum. The binding studies performed in this investigation are concordant with those of Kalkman *et al*. (1986), where [^{125}I]-LSD was used to characterize a postjunctional 5-HT site in guinea-pig ileum. In the study by Kalkman *et al*. (1986), cinanserin (300 nM) was used to mask 5-HT_2 receptors, a concentration which the authors claim theoretically inhibits 98% of 5-HT_2 receptors. Unfortunately, this concentration of cinanserin was also found to occupy close to 50% of the 5-HT_7-like site in a displacement study in the same investigation. This may have led to an underestimation of the K_D value of [^{125}I]-LSD at the 5-HT_7 site.

Nevertheless, the antagonist order of affinity reported (iodo-LSD > metergoline = mesulergine > spiperone > > haloperidol > propranolol) correlates well with a 5-HT_7 receptor.

Despite the lack of binding of [^{3}H]-mesulergine in the rat jejunum, the functional experiments performed in the present study suggest the presence of a 5-HT_7-like receptor in this tissue, in agreement with the findings of McLean & Coupar (1996). The low affinity of pindolol and lack of antagonist affinity of yohimbine, cinanserin, RS 102221 and GR 113808 rule out a 5-HT_{1A}, α_2, 5-HT_{2A}, 5-HT_{2C} and 5-HT_4 receptor site, in contrast to the findings of Javid & Naylor (1997), who reported the involvement of 5-HT_2 and 5-HT_4 receptors in mediating the contractile response of 5-HT in the proximal region of the rat small intestine. The use of ondansetron (1 μM) and the relatively high potency of 5-CT rules out 5-HT_3 receptors. The presence of 5-ht_5 and 5-ht_6 receptors can also be excluded by the potency of 5-CT at this site. The relatively low potency of 5-CT compared to its high affinity in binding studies is characteristic of the 5-HT_7 receptor and was also reported by Carter *et al.* (1995), and Martin & Wilson (1995). The correlation between the pK_B and apparent pK_B values obtained in the rat jejunum and recombinant 5-HT_7 receptors from rat or mouse expressed in transfected cells (Plassat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993; Roth *et al.*, 1994) was very high (see Results).

It is interesting to note that the affinity values of mesulergine (7.3) and ritanserin (7.3) were lower in the present study with the rat isolated jejunum than reported in a similar study by McLean & Coupar (1996), where mesulergine and ritanserin were reported to have pA_2 values of 8.1 and 8.0 respectively. Affinity values for LSD, mesulergine and ritanserin reported in the guinea-pig ileum, receptors expressed from 5-HT_7 receptor genes (Ruat *et al.*, 1993; Carter *et al.*, 1995) and binding studies in the rat brain and guinea-pig ileum in this investigation were also slightly higher than those obtained in the rat jejunum. However, binding studies performed in the guinea-pig brain (To *et al.*, 1994), have shown a pK_D value of 7.8 for LSD, which agrees with the pK_B value obtained in the present study. In addition, risperidone, ritanserin and LSD resulted in a non-competitive antagonism of the serotonin response in the presence of ondansetron (1 μM). At higher concentrations, risperidone (10 – 100 nM) and ritanserin (100 nM – 1 μM) caused a depression of the maximum response to 5-HT without further shifting the control curve. A comparison of the functional characteristics of LSD and risperidone at the 5-HT_7 receptor can not be made, as the effect of these compounds has not been investigated in other studies. However, at a concentration range of 10 – 100 nM, ritanserin was reported to act as a competitive inhibitor of the 5-HT_7-like response in the rat jejunum, (McLean & Coupar, 1996). The observation that an increased concentration of ritanserin and risperidone did not further shift the mean curve to 5-HT may indicate that the interaction of the antagonists with the 5-HT_7 receptor is of a non-competitive nature such as that seen with allosteric modulation. Negative allosteric modulators are known to produce parallel shifts of agonist concentration-response curves up to a limiting value (Ehlert, 1988; Lanzafame *et al.*, 1996). Often, as seen with ritanserin, they may appear to be competitive at lower concentration ranges. Further studies are required to fully define the mechanism by which ritanserin and risperidone interact with the 5-HT_7 site.

The lack of [^{3}H]-mesulergine binding in the rat jejunum in this study may be explained by a low 5-HT_7 receptor density. This is supported by the observation that a response to 5-HT in the rat jejunum after blockade of 5-HT_3 receptors with ondansetron was not always present; (see Results). The response observed in the rat jejunum differs from other smooth muscles where activation of the 5-HT_7 receptor results in relaxation or various preparations in different species, such as porcine vena cava and myometrium (Sumner *et al.*, 1989; Kitazawa *et al.*, 1998), canine coronary artery (Cushing & Cohen, 1992), marmoset aorta (Dyer *et al.*, 1994) guinea-pig ileum (Feniuk *et al.*, 1984; Kalkman *et al.*, 1986; Carter *et al.*, 1995) and cat saphenous vein (Hoyer *et al.*, 1994). In addition, the contractile response is not consistent with the coupling mechanism of the 5-HT_7 receptor, which to date has been shown to only involve activation of adenylate cyclase, (Shenker *et al.*, 1987; Eglen *et al.*, 1997).

5-HT_7 receptor subtypes?

The possibility of 5-HT_7 receptor subtypes cannot be excluded; Ruat *et al.* (1993) isolated a 448 amino acid cDNA in the rat (5-$HT_{7(a)}$), while Lovenberg *et al.* (1993) isolated a 435 amino acid cDNA (5-$HT_{7(b)}$). The difference between the two cDNAs was only in the carboxy-terminal of the seven transmembrane structure of the expressed receptor and was said to result from alternative splicing (Boess & Martin, 1994). The generation of two receptor variants differing in the carboxy regions, can result in differential coupling to G-proteins (Lucas & Hen, 1995) and possibly a different physiological response as has been reported with the prostaglandin EP_3 (Namba *et al.*, 1993) and somatostatin SST_2 receptors (Vanetti *et al.*, 1993). In fact, the neuronal 5-$HT_{7(a)}$ isoform has been reported to increase intracellular calcium resulting in activation of calmodulin-stimulated, adenylate cyclase isoforms AC1 and AC8, independent of phosphoinositide and protein kinase C (Baker *et al.*, 1998). This unique mechanism of coupling for any of the serotonin receptors was reported to be G_s-independent in studies with whole cells (see Wayman *et al.*, 1994; Sunahara *et al.*, 1996). It is possible some similar mechanism may exist in the rat jejunum, where a contraction instead of a relaxation to a 5-HT_7-like receptor was observed.

Furthermore, an additional rat 5-HT_7 isoform, a 470 amino acid cDNA, (5-$HT_{7(c)}$) resulting from a retained exon cassette, has also been reported (Heidmann *et al.*, 1997). However, unlike the other two isoforms, the rat 5-$HT_{7(c)}$ was reported not to be present in human tissue, which suggests a species difference for the presence of various 5-HT_7 isoforms (Heidmann *et al.*, 1997). This again raises the possibility of a difference in the physiological response produced by 5-HT_7 receptors and may explain why an 'atypical' response was obtained in the rat jejunum.

In conclusion binding studies performed with [^{3}H]-mesulergine were able to detect 5-HT_7 sites in rat brain and guinea-pig ileum, but not rat jejunum, where a functional 5-HT_7-like site was present.

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