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Characterisation of a 5-HT$_7$ binding site in mouse ileum

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Abstract

The aim of the present study was to identify 5-hydroxytryptamine$_7$ (5-HT$_7$) binding sites in the mouse ileum, where the presence of mRNA for the receptor has been reported. Studies were performed using [^{3}H]mesulergine, an antagonist with high affinity at 5-HT$_7$ receptors. In the presence of a combination of masking drugs to inhibit the binding of the radioligand to other receptors at which it has affinity, such as 5-HT$_{2A}$, 5-HT$_{2C}$ and dopamine D$_2$ receptors as well as α_1/α_2-adrenoceptors, [^{3}H]mesulergine labelled two sites with pK_D values of 9.7 ± 0.7 and 7.4 ± 0.4 and B_{max} values of 37.2 ± 21.4 and 247.8 ± 62.1 fmol mg protein^{-1}, respectively. Displacement studies also indicated the presence of non-homogenous binding sites, which showed a significant correlation (Pearson correlation factors of 0.91 and 0.85) with the 5-HT$_{2C}$ and 5-HT$_7$ receptors, respectively. Total binding to the 5-HT$_{2C}$ receptor was minimal; < 30% of the total specific receptor binding. The antagonist order of affinity at the greater proportion of receptors was: risperidone (pK_i: 9.7) > lysergic acid diethylamide (LSD; 9.4) > metergoline (7.7) > mesulergine (7.5) > ritanserin (6.3) > pindolol (5.6). This receptor also showed a high affinity for 5-carboxamidotryptamine (5-CT; 10.6) and moderate affinity for (±)-2-dipropyl-amino-8-hydroxy-1,2,3,4,-tetrahydronaphthalene (8-OH-DPAT; 7.2), which is typical of the 5-HT$_7$ receptor profile.

Keywords: 5-HT$_7$ receptor; Ileum, mouse; [^{3}H]Mesulergine

1. Introduction

5-Hydroxytryptamine (5-HT) receptors have been classified into seven distinct families based upon structural, transductional and operational criteria. The most recent additions to the growing number of 5-HT receptor subtypes have been the 5-ht$_5$, 5-ht$_6$ and 5-HT$_7$ receptors, which were identified by molecular cloning techniques. Whilst evidence for the endogenous expression and function of the gene products, which encode the 5-ht$_5$ and 5-ht$_6$ receptors remains unknown, the 5-HT$_7$ receptor is now recognised as the receptor previously classified as being 5-HT$_1$-like and positively coupled to adenylate cyclase (Tsou et al., 1994; Eglen et al., 1997; Saxena et al., 1998).

The 5-HT$_7$ receptor has been cloned from mouse (Plassat et al., 1993), rat (Lovenberg et al., 1993; Ruat et al., 1993), human (Bard et al., 1993) and guinea pig (Tsou et al., 1994). 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 and Cohen, 1992), marmoset aorta (Dyer et al., 1994) guinea pig ileum (Feniuk et al., 1984; Kalkman et al., 1986; Carter et al., 1995) and rat jejunum (McLean and Coupar, 1996; Hemedah et al., 1999). In addition, 5-HT$_7$ binding sites have been found in regions of rat and guinea pig brain (Shenker et al., 1987; Tsou et al., 1994), in particular the limbic system and thalamocortical regions, where it has been suggested that the receptor may have a role in affective behaviours (To et al., 1995; 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).

Due to the lack of selective ligands currently available for studying the 5-HT$_7$ receptor, identification of the receptor has relied on the operational criteria of rank order

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of agonist and antagonist potency derived from 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-HT, 5-methoxytryptamine (5-MeOT), clozapine, lysergic acid diethylamide (LSD), and mesulergine, moderate affinity (pK_i 6–7.9) for (±)-2-dipropyl-amino-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 distinguish it from other closely related receptors such as the 5-HT_{1A} receptor.

Despite the high degree of interspecies homology (95%), there have been reports of apparent species differences in the 5-HT_7 receptor. For instance, activation of the 5-HT_7 receptor in the rat jejunum has been reported to result in a contractile response, which differs from other smooth muscle preparations in various species, where a relaxation has been found to occur (McLean and Coupar, 1996; Hemedah et al., 1999). In addition, the affinity of chlorpromazine for the mouse 5-HT_7 receptor has been reported to be almost two orders of magnitude lower than the affinity of the compound for the rat 5-HT_7 receptor (Sleight et al., 1995b). The possibility of species differences in the 5-HT_7 receptor is supported by the finding of different isoforms in the rat and human (Heidmann et al., 1997).

Hence, the primary aim of this investigation was to identify 5-HT_7 binding sites in the mouse ileum using [^{3}H]mesulergine in order to compare the pharmacological profile of the receptor in intestinal tissue across species. As yet, only mRNA for the 5-HT_7 receptor has been identified in mouse ileum (Plassat et al., 1993).

Although 5-HT_7 sites have been previously labelled using the agonists [^{3}H]-5-CT and [^{3}H]-5-HT (Sleight et al., 1995a; To et al., 1995), [^{3}H]mesulergine, a non-selective antagonist, was chosen as it has a high affinity for 5-HT_7 receptors and has been used successfully in a previous study to label 5-HT_7 sites in the rat brain and guinea pig ileum, (Hemedah et al., 1999). Furthermore, the use of an antagonist radioligand such as [^{3}H]mesulergine avoids some the complications often encountered when binding with an agonist radioligand in tissues where multiple affinity states for the agonist may be present (Kenakin, 1984).

2. Methods

2.1. Membrane preparation

Homogenates were prepared according to the method of Bonhaus et al. (1993), who detected a 5-HT_3 binding site in the mouse ileum. Briefly, mice of either sex were killed by CO_2 asphyxiation before removal of the ileum and flushing out of the intra-luminal contents with phosphate buffer. The segments (ca. 5 cm) were stretched over a wire 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, homogenised in an Ultra Turrax set at 0.75 × maximum speed for 2 × 10 s, and centrifuged at 48,000 × g for 15 min at −2°C. The pellet was re-homogenised, passed through a double layer of coarse mesh gauze and re-centrifuged at 48,000 × g for 15 min. The resulting pellet was again collected, washed with 10 volumes of buffer and re-centrifuged as previously. The final pellet was collected and stored at −80°C until required for radioligand binding studies.

2.2. 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 the above 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. This combination of masking drugs had been used previously in the characterisation of the 5-HT_7 binding site using [^{3}H]mesulergine in the rat brain and guinea pig ileum (Hemedah et al., 1999).

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 Na^+ (Closse, 1983).

Saturation curves were constructed as follows: an aliquot of 200 μl of homogenate containing between 300–500 μg of protein, determined by the Bradford method (Bradford, 1976), was used with various concentrations of the radioligand ranging from 0.3 to 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 composition of the assay medium for competition studies 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 10 different concentrations and the experiment performed in duplicate.

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 have shown no significant difference in specific binding ($P > 0.05$) between experiments incubated for 30, 60 and 90 min, (Hemedah et al., 1999). 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) after at least 5 h had elapsed.

2.3. Drugs

The following drugs were used: 5-CT, cinanserin, LSD, metergoline, (−) pindolol, raclopride L-tartrate, risperidone, (Research Biochemicals International, Natick, MA, USA), prazosin hydrochloride, yohimbine hydrochloride (ICN Biomedicals, Aurora, OH, USA, [^{3}H]mesulergine (Amersham, Amersham, U.K), RS 102221 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulphonamido) phenyl-5-oxopentyl]-1,3,8 triazaspiro[4.5]decane-2,4-dione), 8-OH-DPAT (±)-2-dipropyl-amino-8-hydroxy-1,2,3,4,-tetrahydronaphthalene hydrobromide (Tocris Cookson, Bristol, U.K), ritanserin (Janssen-Cilag, Sydney, Australia), Gpp(NH)p 5′-guanylylimidodiphosphate Na^+ (Sigma, Poole, UK). Ritanserin, pindolol and cinanserin were dissolved in methanol, 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.

2.4. Data analysis

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

$$y = (B_{max} X)/(K_D + X),$$

where B_{max} is the maximum specific binding when a plateau has been reached and K_D is the concentration of radioligand X, required to bind to 50% of receptors.

The equation used for fitting two site saturation curves was:

$$y = [(B_{max1} X)/(K_{D1} + X)] + [(B_{max2} X)/(K_{D2} + X)],$$

where K_{D1} is the concentration of radioligand X, required to bind to 50% of the first population of receptors with maximum binding described by B_{max1} and K_{D2} is the concentration of radioligand X, required to bind to 50% of the second population of receptors with maximum binding described by B_{max2}.

Competition data were analysed according to one or two site displacement models. The equation used for fitting one site competition curves was:

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

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, x, required to inhibit the binding of the radioligand by 50%. K_i values were obtained using the Cheng and Prusoff equation (Cheng and Prusoff, 1973).

The equation used for fitting two site competition curves was:

$$Y = \frac{100(A + B)}{(C + D)}$$

where the constants A, B, C and D are represented by the following:

$$A = \frac{F_1}{1 + (10^{\mathrm{Log}\,K_{D1} - \mathrm{Log}\,R})(1 + 10^{X - \mathrm{Log}\,K_{i(1)}})}$$

$$B = \frac{1 - F_1}{1 + (10^{\mathrm{Log}\,K_{D2} - \mathrm{Log}\,R})(1 + 10^{X - \mathrm{Log}\,K_{i(2)}})}$$

$$C = \frac{F_1}{1 + (10^{\mathrm{Log}\,K_{D1} - \mathrm{Log}\,R})}$$

$$D = \frac{1 - F_1}{1 + (10^{\mathrm{Log}\,K_{D2} - \mathrm{Log}\,R})}$$

and F_1 is the fraction of receptors with an affinity Log $K_{D(1)}$ (the logarithm of the concentration of the radiolabelled

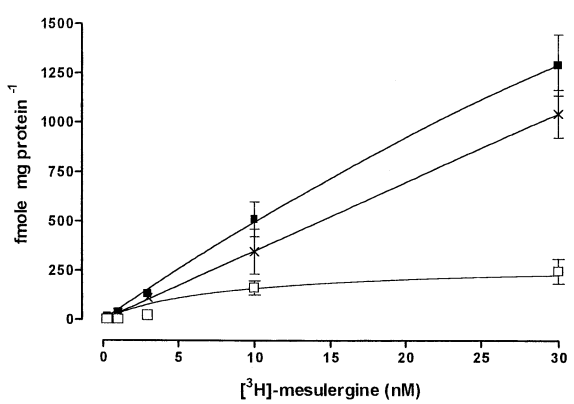

Fig. 1. Total (■), non-specific (X) and specific (□) binding curves of [^{3}H]mesulergine in the mouse intestine in the presence of yohimbine (0.1 μM), prazosin (0.1 μM) cinanserin (30 nM) RS 102221 (3 μM) and raclopride (1 μM). Abscissa: Concentration of [^{3}H]mesulergine in nM; Ordinate: fmole mg of protein^{-1}. Each point is the mean ± S.E.M. from three experiments.

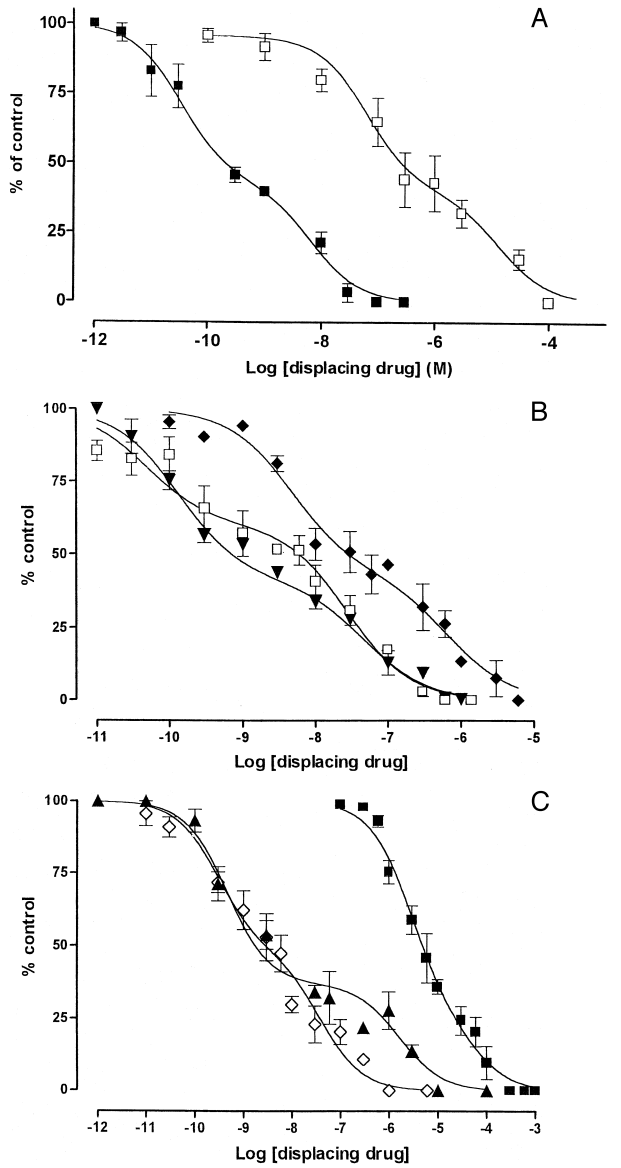

Fig. 2. Inhibition of [^{3}H]mesulergine binding in mouse intestine by (A) 5-CT (■) and 8-OH-DPAT (□), (B) ritanserin (◆), mesulergine (▼) and metergoline (□) and (C) pindolol (■), risperidone (◇) and LSD (▲). Abscissa: Log molar concentration of the displacing drug, Ordinate: percentage of specific binding in the absence of the displacer. The data represent the mean ± S.E.M. percent of maximum specific binding (defined with 1 μM risperidone) of at least three experiments.

drug, Log R, required to bind to 50% of receptor sites) and the remaining receptors have an affinity described by Log $K_{D(2)}$. Student's t-test was used for comparison of individual means; the criteria for statistical significance was set at $P < 0.05$. Arithmetic and geometric means are given with associated S.E.M. or 95% confidence intervals, respectively. Pearson correlation coefficients were calculated using the computer program Graph Pad Prism 2.0 (GraphPad Software, San Diego, CA, USA).

All other calculations and graphics were also performed using Graph Pad Prism 2.0.

3. Results

Saturation studies performed in the mouse ileum to identify 5-HT$_7$ sites, were carried out in presence of cinanserin (30 nM), RS 102221 (3 μM), raclopride (1 μM), prazosin (0.1 μM) and yohimbine (0.1 μM) in an attempt to mask 5-HT$_{2A}$, 5-HT$_{2C}$ receptors, dopamine D$_2$ receptors and α_1/α_2-adrenoceptors, respectively. The concentrations of masking drugs used in the present study were found to be optimum for [^{3}H]mesulergine binding to 5-HT$_7$ receptor binding in the rat brain and guinea pig ileum previously (Hemedah et al., 1999). However saturation experiments undertaken in the current study revealed non-homogenous binding sites for [^{3}H]mesulergine. A two-site binding model better described the data than a one-site model with R^2 values of 0.76 and 0.73, respectively ($P < 0.05$). The pK_D values calculated using the two-site analysis were 9.7 ± 0.7 and 7.4 ± 0.4 with B_{max} values of 37.2 ± 21.4 and 247.8 ± 62.1 fmol mg protein^{-1}, respectively ($n = 3$, Fig. 1).

The binding of [^{3}H]mesulergine to sites other than 5-HT$_7$ receptors in the mouse ileum was also apparent

Table 1
Comparison of ligand affinities in mouse ileum, guinea pig ileum and isolated rat jejunum. All data are expressed as mean ± S.E.M. High and low affinity values as well as the percentage fraction are shown for compounds displaying two site competition

Compound	Mouse ileum pK_i	Guinea pig ileum[a] pK_i or pK_D	Rat jejunum[a] pK_B or apparent pK_B
Agonists			
5-CT	10.6 ± 0.3 (39%) 8.3 ± 0.4 (61%) $n = 4$	12.1 ± 0.5 (46%) 9.4 ± 0.5 (54%) $n = 4$	–
8-OH-DPAT	7.2 ± 0.5 (59%) 5.0 ± 0.6 (41%) $n = 4$	–	
Antagonists			
Risperidone	9.7 ± 0.2 (79%) 8.2 ± 0.2 (21%) $n = 4$	9.4 ± 0.4 $n = 3$	8.9 ± 0.5 $n = 3$
LSD	9.4 ± 0.1 (86%) 6.6 ± 0.2 (14%) $n = 6$	9.4 ± 0.6 $n = 3$	7.6 ± 0.3 $n = 3$
Metergoline	7.7 ± 0.1 (85%) 11.2 ± 0.2 (15%) $n = 3$	8.8 ± 0.3 $n = 3$	7.8 ± 0.3 $n = 3$
Mesulergine	7.5 ± 0.16 (72%) 10.7 ± 0.1 (28%) $n = 7$	7.9 ± 0.3[b] $n = 3$	7.3 ± 0.1 $n = 3$
Ritanserin	6.3 ± 0.2 (74%) 9.2 ± 0.2 (26%) $n = 4$	8.3 ± 0.2 $n = 3$	7.3 ± 1.0 $n = 3$
Pindolol	5.6 ± 0.1 (92%) 5.2 ± 0.45 (8%) $n = 5$	6.3 ± 0.5 $n = 3$	6.7 ± 0.7 $n = 3$

[a] Data obtained from Hemedah et al. (1999).
[b] pK_D value.

from the two-site displacement curves obtained with the antagonists risperidone, ritanserin, mesulergine, metergoline, LSD and pindolol (Fig. 2). In each case, binding to the non-5-HT_7 sites constituted only 8–30% of total specific receptor binding. The antagonist order of affinity at the lower proportion of sites was: metergoline > mesulergine > ritanserin > risperidone > LSD > pindolol (Table 1, Fig. 2). This profile shows a high correlation with the 5-HT_{2C} receptor (Pearson correlation factor 0.91, Fig. 3). In addition, in a separate experiment ($n = 3$), the binding of [^{3}H]mesulergine at 5-HT_{2C} sites in the mouse ileum resulted in a pK_D value of 9.7 ± 0.5 and a B_{max} value of 18.03 ± 9.7 fmol mg protein^{-1} for [^{3}H]mesulergine. In this case, masking drugs consisting of cinanserin (30 nM), 5-CT (0.3 μM), raclopride (1 μM), prazosin (0.1 μM) and yohimbine (0.1 μM) were used to mask 5-HT_{2A}, 5-HT_7 and dopamine D_2 receptors as well as α_1/α_2-adrenoceptors. At the concentrations used, the masking drugs were calculated to block < 30% of 5-HT_{2C} receptors. RS 102221 (3 μM) was used to define non-specific binding.

In contrast to binding at the lower proportion, the antagonist order of affinity at the remaining (greater) proportion of [^{3}H]mesulergine binding sites was: risperidone > LSD > metergoline > mesulergine > ritanserin > pindolol (Table 1, Fig. 2).

The 5-HT_7 agonists, 5-CT and 8-OH-DPAT, compounds with a low (micromolar) affinity at 5-HT_{2C} receptors, also best fitted a two-site competition model, which was not significantly affected by incubation with Gpp(NH)p (100 μM). The correlation between the pK_i values obtained at the latter binding site and previously published values from recombinant 5-HT_7 receptors (obtained from rat or mouse and expressed in transfected cells) was significant ($P < 0.05$); a Pearson correlation factor of 0.85 was obtained (Fig. 3).

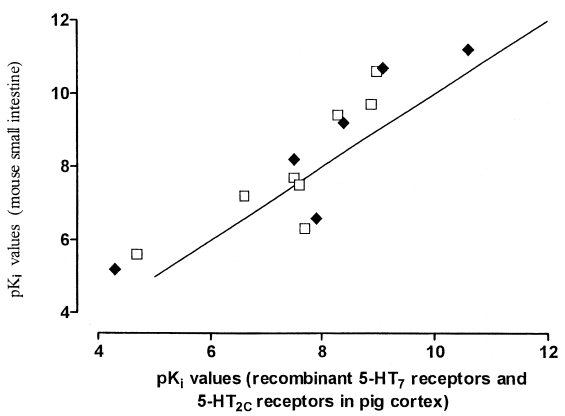

Fig. 3. Correlation between pK_i values obtained in either recombinant 5-HT_7 receptors from rat or mouse and expressed in transfected cells (Plassat et al., 1993; Ruat et al., 1993; Shen et al., 1993; Roth et al., 1994) and the pK_i values obtained in the current study in mouse intestine (□) or between pK_i values obtained from 5-HT_{2C} receptors in pig cortex (Pazos et al., 1985) and the pK_i values obtained in the current study in mouse intestine (◆). Abscissa: pK_i values for recombinant 5-HT_7 receptors and pig cortex 5-HT_{2C} receptors, Ordinate: pK_i values obtained in mouse intestine. The continuous line represents a line of identity.

4. Discussion

The results from the current study suggest the presence of a 5-HT_7 receptor binding site in the mouse ileum, in agreement with the findings of Plassat et al. (1993), where mRNA of the 5-HT_7 receptor was identified.

4.1. Receptor characterisation

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 characterise the 5-HT_7 site, as has been undertaken previously in studies using expressed and native 5-HT_7 receptors (Kalkman et al., 1986; Erlander et al., 1993; Monsma et al., 1993; Plassat et al., 1993; Carter et al., 1995; To et al., 1995; McLean and Coupar, 1996, Hemedah et al., 1999).

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 receptors 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 targeted 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. Studies using the same protocol have been successfully undertaken in the characterisation of the 5-HT_7 site in the rat brain and guinea pig ileum (Hemedah et al., 1999).

Saturation studies performed in the mouse small ileum using the combination of masking drugs outlined above revealed non-homogenous binding sites for [^{3}H]mesulergine with pK_D values of 9.7 ± 0.7 and 7.4 ± 0.4. These values are circa the affinity values reported for mesulergine at 5-HT_{2C} and 5-HT_7 sites respectively. Previously reported pK_D values ranging from 8.7 to 9.1 have been noted for [^{3}H]mesulergine at 5-HT_{2C} receptors (Pazos et al., 1985; Hoyer et al., 1994). A range of affinity values have also been noted for mesulergine at 5-HT_7 receptors: in cells transfected with the mouse 5-HT_7 receptor a pK_i value of 7.6 was obtained (Plassat et al., 1993), whilst a pK_B value of 7.8 (Carter et al., 1995) and a pK_i value of 8.2 (Hoyer et al., 1994; To et al., 1995) have been reported at the native and recombinant 5-HT_7 receptor.

The binding of [^{3}H]mesulergine to 5-HT$_{2C}$ and 5-HT$_7$ receptors in the mouse ileum was also apparent from the two-site displacement curves obtained with the antagonists risperidone, ritanserin, mesulergine, metergoline, LSD and pindolol used. In each case, binding to the non-5-HT$_7$ sites constituted between 8–30% of the total receptor population. The antagonist order of affinity at the lower proportion of sites was: metergoline (pK_i:11.2) > mesulergine (9.7) > ritanserin (9.2) > risperidone (8.2) > LSD (6.6) > pindolol (5.2). This profile shows a high correlation with the 5-HT$_{2C}$ receptor (Pearson correlation factor 0.91) and therefore binding to 5-HT$_{2C}$ receptors cannot be excluded, although the selective antagonist RS 102221 (3 μM) was used as a masking agent. Preliminary studies also indicated the presence of a 5-HT$_{2C}$ binding site, where [^{3}H]mesulergine was found to have a pK_D value of 9.7 ± 0.5, which is concordant with the pK_D value obtained at the lower proportion of receptors.

The contribution of the 5-HT$_{2B}$ receptor, which has been cloned from mouse intestine (Boess and Martin, 1994), in the binding of [^{3}H]mesulergine to this lower proportion of binding sites cannot be easily discounted; the presence of yohimbine in the cocktail of masking drugs is sufficient to block ca. 70% of these 5-HT$_{2B}$ sites, leaving about 30% ''free'' to bind to [^{3}H]mesulergine. However, given the affinity values obtained at the lower proportion of sites and the highly significant correlation obtained between this site and the 5-HT$_{2C}$ receptor, any 5-HT$_{2B}$ receptor involvement seems unlikely.

The antagonist order of affinity at the remaining (greater) proportion of receptors was: risperidone (9.7) > LSD (9.4) > metergoline (7.7) > mesulergine (7.5) > ritanserin (6.3) > pindolol (5.6). The pK_i values of these compounds compared well with those from 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).

The 5-HT$_7$ agonists 5-CT and 8-OH-DPAT, which only have a low (micromolar) affinity at 5-HT$_{2C}$ receptors also displayed two-site displacement binding. The latter is not unusual for agonists, since they can bind to both a high and low affinity state of the receptor (Kenakin, 1984). Incubation with Gpp(NH)p (100 μM) did not alter the binding of either agonist under the conditions used indicating that the receptor in question is either insensitive to Gpp(NH)p or that the conditions used were not optimum for detection of a G-protein interaction. The detection of G-protein interactions in binding assays is dependent on a number of factors including pH, temperature and ion concentration (Aronstam and Narayanan, 1988). Interestingly, it has been noted that the optimal temperature for detecting nucleotide sensitivity with muscarinic receptors is between 16–20°C (Aronstam and Narayanan, 1988). Despite this, the existence of Gpp(NH)p insensitive multiple receptor states have been reported for other receptor types such as dopamine D$_2$ receptors (Wreggert and Seeman, 1983) and 5-HT$_2$ receptors expressed in human embryonic kidney (HEK) 293 cells (Szele and Pritchett, 1993).

There was a significant correlation (Pearson correlation factor of 0.85) between the displacement values obtained in this study for the agonists and antagonists investigated and the values reported for the 5-HT$_7$ receptor.

The binding site identified in the mouse 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 and Coupar, 1996). The other 5-HT$_1$ receptor subtypes; 5-ht$_{1E}$, and 5-ht$_{1F}$ were ruled out by the high affinity of 5-CT (Hoyer et al., 1994). Whilst the 5-HT$_{1D}$ receptor is reported to have a relatively high affinity for 5-CT, this is at least 30-fold lower than that seen at the 5-HT$_7$ receptor (Hoyer et al., 1994), which also excludes the involvement of this subtype. Furthermore, the affinity of LSD obtained in the present study at the greater proportion of sites is about 100-fold greater than that reported at the 5-HT$_{1D}$ receptor (Boess and Martin, 1994).

The receptor is also unlikely to be the 5-HT$_{2A}$ subtype firstly because it was excluded with cinanserin, and more importantly the antagonist order of affinity of risperidone ($pK_i = 9.7$), metergoline (7.7) and ritanserin (6.3), respectively, obtained in the mouse ileum did not correlate with the 5-HT$_{2A}$ (risperidone > ritanserin > metergoline) (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 for 5-HT$_{2A}$ receptor sites (Bard et al., 1993; Hoyer et al., 1994; Roth et al., 1994).

The 5-HT$_{2B}$ receptor can also be excluded from the binding site representing the greater proportion of receptors, since 5-CT has been reported to have only a low (micromolar) affinity at this site (Boess and Martin, 1994).

The high affinity of 5-CT and the moderate affinity of 8-OH-DPAT also ruled out the involvement of 5-HT$_3$ receptors, where the agonists are inactive below a pK_i of 4.9 (Zifa and Fillion, 1992; 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 (Erlander et al., 1993; Monsma et al., 1993; Plassat 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).

The possibility of [^{3}H]mesulergine binding to non-serotonergic receptors, such as dopamine receptors, was

ruled out by the addition of masking drugs such as raclopride (Rinne, 1983) and by the use of the agonist 5-CT, which is specific for serotonin receptors.

4.2. Comparison with intestinal tissue from other species

5-HT_7 receptors have been identified in intestinal tissue in a number of species: binding studies have demonstrated the presence of a 5-HT_7 site in the guinea pig ileum (Kalkman et al., 1986; Hemedah et al., 1999), which was shown to mediate relaxation of the pre-contracted tissue (Feniuk et al., 1984; Carter et al., 1995; Kalkman et al., 1986). Conversely, a contractile response to a 5-HT_7-like receptor was seen to occur in the isolated rat jejunum (McLean and Coupar, 1996; Hemedah et al., 1999), although no binding sites could be detected for the latter presumably because of the low concentration of such receptors (Hemedah et al., 1999).

It is interesting to compare the ligand affinities obtained in the current study to those obtained in the guinea pig ileum and rat jejunum (Table 1). Striking differences can be seen with the affinity values of metergoline, ritanserin and LSD across the different species. Metergoline exhibited a ca. 10-fold higher affinity in the guinea pig than in the rat or mouse, whilst ritanserin had a 100-fold lower affinity in the mouse than the guinea pig and a 10-fold lower affinity than in the rat jejunum. The affinity of LSD was ca. 6-fold lower in the rat jejunum than either the mouse or the guinea pig ileum. Such differences in the affinity of compounds at 5-HT_7 receptors in various species are not uncommon; others include the reduced ability of methiothepin to antagonise adenylyl cyclase in the guinea pig compared to the mouse, the 10-fold greater affinity of dihydroergotamine for the human 5-HT_7 receptor than for the rat homologue and the greater affinity of chlorpromazine for the rat 5-HT_7 receptor than for the mouse homologue (Sleight et al., 1995b). These results are not surprising given the existence of different 5-HT_7 isoforms in various species. For instance, the 5-$HT_{7(c)}$ isoform is found in rat but not human tissue, and the 5-$HT_{7(d)}$ isoform is found only in human tissue (Heidmann et al., 1997).

Given these interspecies differences, it would be of interest to determine the functional role of the 5-HT_7 receptor in human and mouse ileum and whether it plays a role in conditions such as irritable bowel syndrome where the possible involvement of other 5-HT receptors has been reported (Sanger, 1996). The advent of knock-out mice deficient in 5-HT_7 receptors may make the physiological role of this relatively novel receptor clearer in the gastrointestinal tract and central nervous system.

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