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Functional characterisation of the human cloned 5-HT_7 receptor (long form); antagonist profile of SB-258719

[1,4]David R. Thomas, [1]Susan A. Gittins, [1]Lissa L. Collin, [1]Derek N. Middlemiss, [1]Graham Riley, [1]Jim Hagan, [2]Israel Gloger, [2]Catherine E. Ellis, [3]Ian T. Forbes & [1]Anthony M. Brown

[1]Departments of Neuroscience Research, [2]Expression Sciences and [3]Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW

1 The functional profile of the long form of the human cloned 5-HT_7 receptor (designated h5-$HT_{7(a)}$) was investigated using a number of 5-HT receptor agonists and antagonists and compared with its binding profile. Receptor function was measured using adenylyl cyclase activity in washed membranes from HEK293 cells stably expressing the recombinant h5-$HT_{7(a)}$ receptor.

2 The receptor binding profile, determined by competition with [^{3}H]-5-CT, was consistent with that previously reported for the h5-$HT_{7(a)}$ receptor. The selective 5-HT_7 receptor antagonist SB-258719 ((R)-3,*N*-Dimethyl-*N*-[1-methyl-3-(4-methylpiperidin-1-yl)propyl]benzene sulfonamide) displayed high affinity (pK_i 7.5) for the receptor.

3 In the adenylyl cyclase functional assay, 5-CT and 8-OH-DPAT were both full agonists compared to 5-HT and the rank order of potency for agonists (5-CT > 5-HT > 8-OH-DPAT) was the same in functional and binding studies.

4 Risperidone, methiothepin, mesulergine, clozapine, olanzapine, ketanserin and SB-258719 antagonised surmountably 5-CT-stimulated adenylyl cyclase activity. Schild analysis of the antagonism by SB-258719 gave a pA_2 of 7.2 ± 0.2 and slope not significantly different from 1, consistent with competitive antagonism.

5 The same antagonists also inhibited basal adenylyl cyclase activity with a rank order of potency in agreement with those for antagonist potency and binding affinity. Both SB-258719 and mesulergine displayed apparent partial inverse agonist profiles compared to the other antagonists tested. These inhibitory effects of antagonists appear to be 5-HT_7 receptor-mediated and to reflect inverse agonism.

6 It is concluded that in this expression system, the h5-$HT_{7(a)}$ receptor shows the expected binding and functional profile and displays constitutive activity, revealing inverse agonist activity for a range of antagonists.

Keywords: Human 5-$HT_{7(a)}$ receptor; long splice variant; functional characterisation; adenylyl cyclase; [^{3}H]-5-CT binding; constitutive receptor activity; inverse agonism; SB-258719

Introduction

5-HT receptors have been divided into seven major classes (5-HT_{1-7}) based on their structural, functional and pharmacological characteristics. Among these receptors 5-HT_4, 5-ht_6 and 5-HT_7 receptors couple positively to adenylyl cyclase when expressed in cell lines (Hoyer *et al*., 1994). The 5-HT_7 receptor can be distinguished pharmacologically from 5-HT_4 and 5-ht_6 receptors by its high affinity for 5-CT and the antipsychotic risperidone, and its moderate affinity for 8-OH-DPAT (Bard *et al*., 1993; Roth *et al*., 1994). To date, the 5-HT_7 receptor has been cloned from mouse, rat, guinea-pig and human and the receptor binding profile appears consistent across species (Plassat *et al*., 1993; Ruat *et al*., 1993; Tsou *et al*., 1994; Bard *et al*., 1993) and also between cloned and native 5-HT_7 receptors (To *et al*., 1995; Boyland *et al*., 1996).

Messenger RNA distribution studies in various species (including man) have shown the 5-HT_7 receptor to be present both centrally and peripherally (e.g. Bard *et al*., 1993). The role of the 5-HT_7 receptor in the CNS has not been fully established, in part due to the lack of selective ligands. However, it has been proposed to play a role in circadian rhythm control (Lovenberg *et al*., 1993) and in the pathophysiology of depression (Sleight *et al*., 1995) and schizophrenia (Roth *et al*., 1994). In the periphery, 5-HT_7 receptors appear to mediate relaxation of smooth muscle, particularly in a number of vascular tissues (Schoeffter *et al*., 1996; Eglen *et al*., 1997).

Long and short splice variants of the human 5-HT_7 receptor have been identified (and designated h5-$HT_{7(a)}$ and h5-$HT_{7(b)}$ respectively) which display a similar receptor binding profile and tissue distribution and which stimulate adenylyl cyclase when expressed in cell lines (Bard *et al*, 1993; Stam *et al*., 1997; Jasper *et al*., 1997). A second short form of the human receptor has also recently been identified (designated h5-$HT_{7(d)}$) (Heidman *et al*., 1997) although no pharmacological studies on this variant have been reported. To date, the functional profile of the h5-$HT_{7(a)}$ receptor has not been extensively characterised, although Bard *et al*. (1993) reported that methiothepin antagonised the 5-HT-induced stimulation of cAMP production in COS-7 cells transiently expressing this form of the h5-HT_7 receptor. More recently, Jasper *et al*. (1997) reported that the rank order of potency for agonists and antagonists at the h5-$HT_{7(b)}$ receptor stably expressed in HEK 293 cells correlated with the order of binding affinity for this splice variant and was similar to that for the h5-$HT_{7(a)}$ receptor.

In the present study we have investigated the receptor binding and functional profile of the h5-$HT_{7(a)}$ receptor using a number of standard 5-HT receptor agonists and antagonists. In addition, we have investigated further the profile of action

[4] Author for correspondence.

of SB-258719, which has recently been reported to be a selective 5-HT$_7$ antagonist displaying at least 100 fold selectivity in receptor binding studies for the human cloned 5-HT$_7$ receptor versus other cloned 5-HT receptor subtypes (Forbes *et al*., 1998). We show that SB-258719 and a number of non-selective 5-HT$_7$ antagonists display profiles consistent with both competitive antagonism and inverse agonism at the cloned h5-HT$_{7(a)}$ receptor.

Methods

Isolation and expression of the gene for the human 5HT$_{7(a)}$ receptor

The full-length 5-HT$_{7(a)}$ receptor gene was obtained by polymerase chain reaction from a human brain cDNA library, subcloned into a mammalian expression vector, pCDNA3 (Invitrogen), and stably expressed in human embryonic kidney cells (HEK293). A clone was selected expressing the 5-HT$_{7(a)}$ receptor at high density. [^{3}H]-5-CT saturation binding studies to the 5-HT$_{7(a)}$/HEK293 membranes gave a B_{max} of 4.4 pmoles mg^{-1} protein (equivalent to 105,000 receptors cell^{-1}, *cf*. Boyland *et al*., 1996) and K_d of 0.5 nM. These data are in good agreement with those previously reported by Boyland *et al*. (1996) for [^{3}H]-5-CT binding to the same 5-HT$_{7(a)}$/HEK293 clone.

Cell culture and preparation of cell membranes

HEK293 cells stably expressing the human 5-HT$_{7(a)}$ receptor were grown in Minimum Essential Medium (MEM) containing 10% dialysed foetal calf serum (Gibco BRL), G418 sulphate (1 mM), glutamine (2 mM) and non-essential amino acids (1%). Cells were grown to confluence, washed with phosphate buffered saline (PBS), and pelleted by centrifugation (1000 g) in PBS containing EDTA (0.1 mM) and dithiothreitol (1 mM). Pellets were stored at −80°C prior to membrane preparation. For preparation of membranes, cell pellets were washed twice by homogenisation (Polytron, 15 s, setting 5) and centrifugation (50,000 g, 15 min, 4°C) in 20 volumes of Tris HCl (25 mM pH 7.4) containing EDTA (0.1 mM). Membranes were then resuspended in buffer and incubated (37°C, 20 min). Following centrifugation and a further wash at 4°C, the membranes were finally re-suspended at a membrane concentration equivalent to 2.5×10^7 cells ml^{-1} and stored at −80°C prior to use.

Radioligand binding

5-HT$_{7(a)}$/HEK293 membranes (8–10 μg protein/tube) were incubated in Tris HCl buffer (50 mM, pH 7.4 at 37°C) containing $CaCl_2$ (4 mM), pargyline (0.1 mM) and ascorbic acid (1 mM) in the presence of [^{3}H]-5-CT (0.5 nM) and with or without test drugs. Incubation (1 h at 37°C) was started by addition of membranes and stopped by rapid filtration through Whatman GF/B grade filters (pre-soaked with 0.3% polyethyleneimine) followed by 4 × 1 ml ice-cold buffer washes. Bound radioactivity was determined by liquid scintillation counting. Non-specific binding was defined in the presence of 10 μM 5-HT.

Adenylyl cyclase assay

Adenylyl cyclase activity in 5-HT$_{7(a)}$/HEK293 membranes was determined by measuring the conversion of [α-^{33}P]-ATP to [^{33}P]-cAMP. The reaction was performed in Tris HCl buffer (25 mM) containing $MgCl_2$ (5 mM), GTP (50 μM), ATP (150 μM), phosphocreatine (20 mM), creatine phosphokinase (40 units/ml), myokinase (50 units/ml), 1-methyl-3-isobutylxanthine (IBMX) (0.5 mM), pargyline (10 μM) and ascorbic acid (0.2 mM) in a total assay volume of 50 μl. Incubations (37°C, 15 min) were started by addition of 20 μl of membrane suspension (20–25 μg protein) to tubes containing incubation buffer, [α-^{33}P]-ATP (1–1.5 μCi/tube, specific activity 2000 Ci/mmol) and test drugs where appropriate. Incubation was stopped by addition of 100 μl of 0.5 M HCl containing ATP (40 mM), cAMP (10 mM) and [^{3}H]-cAMP (~10,000 d.p.m. per assay tube, specific activity 27 Ci/mmol) for calculation of column recovery. Tubes were stored on ice prior to isolating [^{33}P]-cAMP by double-column chromatography according to the method of Salomon (1979). Samples were counted using a dual label protocol and the tritium signal was used to correct for % column recovery.

Measurement of [^{35}S]-GTPγS binding

[^{35}S]-GTPγS binding to 5-HT$_{7(a)}$/HEK293 membranes was measured using the method of Thomas *et al*. (1995a). Briefly, membranes (20–30 μg protein) were preincubated (30°C for 30 min) in 20 mM HEPES buffer (pH 7.4) in the presence of 3 mM $MgCl_2$, 100 mM NaCl, 10 μM GDP, 0.2 mM ascorbate and in the presence or absence of test drugs. Incubations (30 min, 30°C) were started by addition of [^{35}S]-GTPγS (0.1–3 nM) followed by vigorous mixing and stopped by rapid filtration through Whatman GF/B filters followed by five 1 ml washes with ice-cold buffer containing 20 mM HEPES and 3 mM $MgCl_2$. All determinations within an experiment were performed in duplicate. Radioactivity on the filters was determined using liquid scintillation spectrometry.

Measurement of 5-HT concentrations in cell culture media

5-HT concentrations in cell culture media were measured by HPLC with electrochemical detection using a method similar to that described by Hutson *et al*. (1991). The limit of detection in this system was 1–2 nM.

Data analysis

The concentration of drug inhibiting specific [^{3}H]-5-CT binding by 50% (IC_{50}) was determined by iterative curve fitting (Bowen and Jerman, 1995). pK_i values (−log of the inhibition constant) for receptor binding were then calculated from the IC_{50} values as described by Cheng and Prusoff (1973) using a K_d value of 0.5 nM.

Drug concentration-response curves from adenylyl cyclase experiments were analysed by a four parameter logistic equation using GRAFIT (Erithacus Software), and drug potency expressed as the pEC_{50} or pIC_{50} (−log EC_{50} and −log IC_{50} respectively) for stimulation or inhibition of the basal response. Non-enzymic [^{33}P]-cAMP production (measured at 4°C) was found to be less than 1% of the basal activity (measured at 37°C). Apparent pK_B values for antagonism (−log of the antagonist equilibrium dissociation constant) were determined using the equation: (pK_B = (−log ([antagonist]/(concentration ratio-1)) where concentration ratio = ratio of the agonist EC_{50}*s* in the presence and absence of antagonist). Since all the antagonists tested reduced basal cyclase activity, agonist curves generated in the presence of

Table 1 Drug effects on basal and 5-CT-stimulated adenylyl cyclase activity and comparison with binding affinities

Compound	pK_i[a]	pK_B[b]	*pEC_{50}[c] or pIC_{50}	% inhibition of basal[d]
5-CT	9.1±0.1	–	*7.7±0.1	–
5-HT	8.2±0.1	–	*6.8±0.1	–
8-OH-DPAT	6.6±0.1	–	*6.1±0.1	–
Risperidone	8.3±0.1	8.3±0.1 (0.1)	7.6±0.1	86±2.6
Methiothepin	8.5±0.1	8.1±0.1 (1)	7.3±0.2	82±3.4
Mesulergine	7.5±0.1	7.5±0.2 (1)	7.1±0.1	49±5.2
Clozapine	7.2±0.1	7.2±0.1 (1)	6.6±0.1	79±4.3
SB-258719	7.5±0.1	7.0±0.1 (3)	6.6±0.2	37±1.0
Olanzapine	6.5±0.1	6.3±0.1 (10)	6.3±0.1	88±1.7
Ketanserin	6.1±0.1	6.3±0.1 (10)	6.0±0.1	77±10

[a] pK_i (-log inhibition constant) from [^{3}H]-5-CT binding experiments; [b] pK_B (-log antagonist equilibrium dissociation constant) calculated from the antagonism of 5-CT-stimulated adenylyl cyclase activity. The concentration of drug tested (μM) is shown in parentheses; [c] pEC_{50} (-log EC_{50}) or pIC_{50} for stimulation or inhibition of basal adenylyl cyclase activity respectively; [d]% inhibition of basal adenylyl cyclase activity for antagonists, measured in the absence of added agonist. Data are the mean±s.e. mean from at least three separate experiments each performed in duplicate.

antagonists were fitted to their respective minima and maxima. The antagonist concentration used was chosen to be between 10 and 100 fold greater than the K_i from radioligand binding. Data for antagonism by SB-258719 (Figure 4) were analysed using a Schild plot (Arunlakshana and Schild, 1959) and an estimate of the pA_2 obtained.

Data are expressed as the mean±s.e.mean of at least three separate experiments and, except where stated, all determinations within an experiment were performed in duplicate.

Drugs

5-hydroxytryptamine HCl (5-HT) was obtained from Sigma (St Louis, U.S.A.). 5-carboxamidotryptamine (5-CT), olanzapine and SB-258719 ((R)-3,*N*-Dimethyl-*N*-[1-methyl-3-(4-methylpiperidin-1-yl) propyl] benzenesulfonamide) were synthesised at SmithKline Beecham (Harlow, U.K.). 8-OH-DPAT, methiothepin mesylate, mesulergine hydrochloride, clozapine and ketanserin tartrate were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). [α-^{33}P]-ATP and [^{3}H]-cAMP were obtained from NEN Du Pont. [^{3}H]-5CT was obtained from Amersham (U.K.). Stock drug solutions were prepared fresh on the day of assay in de-ionised water or DMSO (the final assay concentration of DMSO not exceeding 0.4%). Drug dilutions were prepared in 25 mM Tris buffer (pH 7.7 at 25°C) containing 0.2 mM ascorbic acid.

Results

Receptor binding

The overall binding profile for standard 5-HT receptor ligands was consistent with that previously reported for the 5-HT$_7$ receptor (Bard *et al*., 1993; Roth *et al*., 1994) (Table 1). For example, 5-CT and risperidone showed high affinity whereas 8-OH-DPAT and ketanserin showed lower affinity for the receptor. The selective antagonist SB-258719 displayed high affinity (pK_i 7.5).

[^{35}S]-GTPγS binding

[^{35}S]-GTPγS binding to 5-HT$_{7(a)}$/HEK293 membranes was initially investigated as a potential means of assessing receptor function. Basal [^{35}S]-GTPγS binding was not stimulated by 5-CT (0.1 and 1 μM) using a range of concentrations of [^{35}S]-GTPγS (0.1–3 nM) (data not shown).

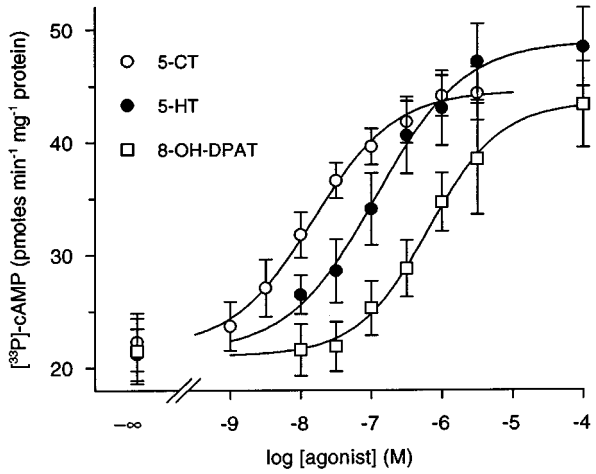

Figure 1 Stimulation of adenylyl cyclase activity in human 5-HT$_7$/HEK293 membranes by 5-CT, 5-HT and 8-OH-DPAT. Data points represent the mean±s.e.mean of three separate experiments each performed using duplicate determinations.

Adenylyl cyclase functional assay

An adenylyl cyclase assay was used to characterise the functional activity of the human 5-HT$_{7(a)}$ receptor expressed in HEK293 cells. In an initial batch of 5-HT$_{7(a)}$/HEK293 membranes, 5-HT stimulated adenylyl cyclase activity from a basal level of 21±2 pmoles mg^{-1} protein to 48±4 pmoles mg^{-1} protein (129% stimulation) with a pEC_{50} of 6.8±0.1 (Figure 1, Table 1). Subsequent membrane batches showed some variation in the absolute levels of basal and 5-HT-stimulated adenylyl cyclase activity. However, the relative levels of basal activity and 5-HT stimulated activity, and degree of inverse agonism were consistent between batches, as was the overall pharmacological profile. 5-CT and 8-OH-DPAT were full agonists compared to 5-HT (Figure 1) with a rank order of potency (5-CT > 5-HT > 8-OH-DPAT) corresponding to the order of agonist binding affinity. Absolute functional potencies were somewhat lower than the corresponding binding affinities (Table 1). No stimulation of adenylyl cyclase activity by 5-CT (10 μM) was detected using membranes prepared from untransfected (parental) HEK 293 cells (data not shown).

A number of non-selective 5-HT$_7$ receptor antagonists, namely methiothepin, mesulergine, ketanserin, and the

antipsychotic compounds risperidone, clozapine and olanzapine were investigated for their effects on basal and 5-CT-stimulated adenylyl cyclase activity. These antagonists all antagonised surmountably 5-CT-stimulated adenylyl cyclase activity. Figure 2 shows representative data for methiothepin (1 μM), mesulergine (1 μM) and ketanserin (10 μM). SB-258719 (tested at 1, 3 and 10 μM) produced a concentration-related rightward-shift of the 5-CT concentration-response curve with no significant alteration in the maximal response to 5-CT (Figure 3). Schild analysis of the data for SB-258719 gave a pA_2 of 7.2$\pm$0.2 and slope (0.82, 95% confidence interval 0.57–1.06) which was not significantly different from 1, consistent with competitive antagonism (Figure 3, inset). This pA_2 is in good agreement with the pK_B determined using 3 μM SB-258719 (Table 1). The overall rank order of antagonist potency (pK_B) for these compounds was in good agreement with the order of binding affinity (Table 1; Pearson's product moment linear correlation coefficient = 0.96, $P<0.001$). In addition, for each antagonist, absolute values for antagonist potency and binding affinity were similar (Table 1).

In addition to antagonising the effect of 5-CT, the above compounds also reduced basal cyclase activity (Figures 2, 3) and this effect was investigated further. All of the antagonists studied produced a concentration-related inhibition of basal activity. Figure 4 shows the effects of methiothepin, clozapine mesulergine, ketanserin and SB-258719. The rank order of potency (pIC_{50}) to inhibit basal activity was in agreement with that of antagonist potency (pK_B) and of binding affinity (pK_i) (Table 1; Pearson's product moment linear correlation coefficients were 0.97 ($P<0.001$) and 0.94 ($P<0.005$) respectively), although absolute pIC_{50} values were generally lower than the corresponding pK_B values. The maximal degree of inhibition of basal activity varied with different compounds. Risperidone, methiothepin, clozapine, olanzapine and ketanserin produced 77–88% inhibition, whereas mesulergine and SB-258719 produced significantly less inhibition (Table 1 and Figure 4) (49% and 37% respectively, $P<0.01$, Student's 't'-test, compared to the maximal inhibition produced by methiothepin). No inhibition of basal activity by clozapine (10 μM) could be detected using membranes prepared from untransfected (parental) HEK293 cells (data not shown), consistent with the inhibitory effects being 5-HT$_7$ receptor-mediated.

A number of studies were carried out to investigate the mechanism of inhibition of basal activity. Membranes used in the adenylyl cyclase assays were prepared from cells grown in medium containing dialysed serum, raising the possibility that the inhibitory effects seen might reflect antagonism of residual endogenous 5-HT carried over from the cell growth medium. This seems unlikely. The concentration of 5-HT in the cell growth medium, measured by HPLC, was only 4$\pm$1 nM, so that, following membrane preparation (see methods), the concentration of 5-HT carried over into the adenylyl cyclase assays would be expected to be subnanomolar, well below the EC_{50} for 5-HT in the assay (Table 1). The effectiveness of the membrane washing procedure in removing potential contaminating 5-HT was confirmed in experiments where unwashed cell homogenates were preincubated with 5-HT (100 nM) prior to carrying out the standard washing/incubation procedure. Comparison of responses using these membranes with those for control membranes showed no significant differences in the levels of either basal or 5-CT (10 μM)-stimulated adenylyl cyclase activity (data not shown). These findings suggest that endogenous 5-HT is unlikely to have been present at a level sufficient to contribute to the measured basal adenylyl cyclase activity through stimulation of 5-HT$_7$ receptors (see Discussion).

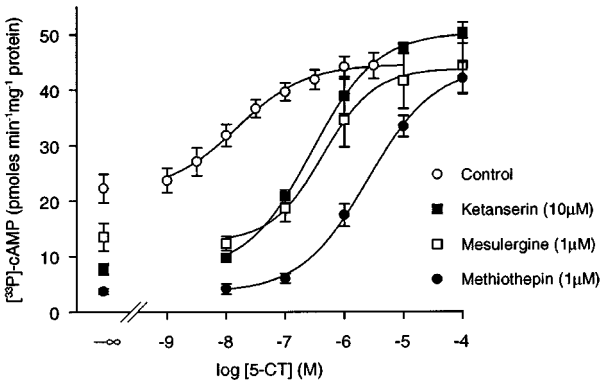

Figure 2 Stimulation of adenylyl cyclase activity in human 5-HT$_7$/HEK293 membranes by 5-CT alone and in the presence of ketanserin (10 μM), mesulergine (1 μM), and methiothepin (1 μM). Data points represent the mean$\pm$s.e.mean of three separate experiments each performed using duplicate determinations.

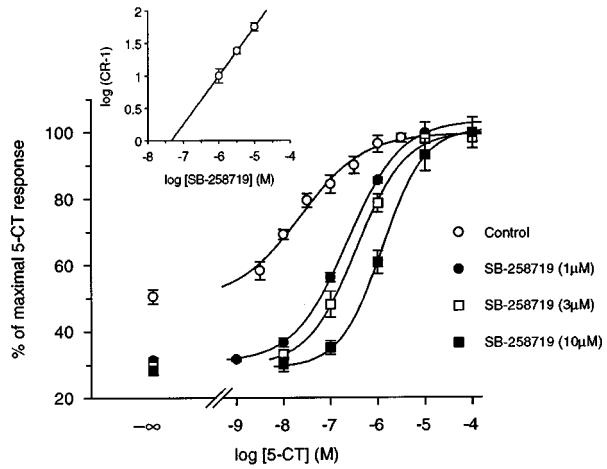

Figure 3 Stimulation of adenylyl cyclase activity in human 5-HT$_7$/HEK293 membranes by 5-CT alone and in the presence of SB-258719 (1, 3 and 10 μM). Data points represent the mean$\pm$s.e.mean of at least three separate experiments each performed using duplicate determinations. Inset: Schild analysis of the same data.

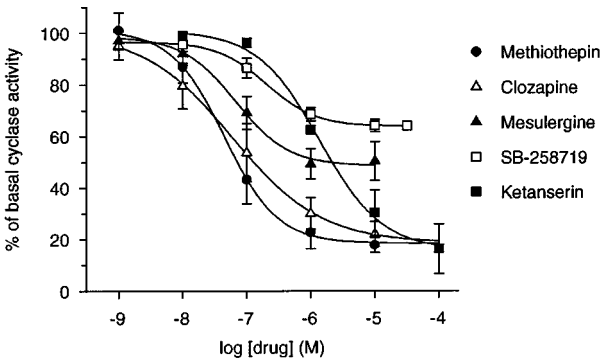

Figure 4 Inhibition of basal adenylyl cyclase activity in human 5-HT$_7$/HEK293 membranes by methiothepin, clozapine, mesulergine, SB-258719, and ketanserin. Data points represent the mean$\pm$s.e.mean of at least three separate experiments each performed using duplicate determinations.

Discussion

The long form of the human cloned 5-HT$_7$ receptor (recently designated h5-HT$_{7(a)}$) has been well characterised in terms of its receptor binding profile (Bard *et al*., 1993), but its functional activity has not been studied in detail. In the present study, we have used [^{3}H]-5-CT binding and adenylyl cyclase activity in washed membranes to investigate and compare the binding and functional profiles of the human cloned 5-HT$_{7(a)}$ receptor stably expressed in HEK293 cells. We have also investigated further the profile of action of the selective 5-HT$_7$ receptor antagonist SB-258719 in this system.

The receptor binding profile for the human 5-HT$_{7(a)}$ receptor was consistent with that reported previously (Bard *et al*., 1993), with 5-CT and methiothepin showing high affinity for the receptor and 8-OH-DPAT and ketanserin showing lower affinity. In addition, the affinity of 5-HT (pK$_i$ 8.2) was lower than that for 5-CT (pK$_i$ 9.1), as reported by Bard *et al*. (1993). This contrasts with the data of Jasper *et al*. (1997) who reported similar affinities for 5-HT and 5-CT at the human 5-HT$_{7(a)}$ receptor.

[^{35}S]-GTPγS binding to 5-HT$_{7(a)}$/HEK293 membranes was initially investigated as a potential simple means of assessing receptor function. However, basal binding was not stimulated by 5-CT (0.1 and 1 μM) using a range of concentrations of [^{35}S]-GTPγS (100 – 3000 pM). The reason for the inability to detect a 5-HT$_7$ receptor mediated stimulation of binding in this system is unclear, although stimulation of [^{35}S]-GTPγS binding is generally reported with receptors which couple via G$_i$ but less often in G$_s$-coupled systems. Subsequent functional studies were therefore carried out using the adenylyl cyclase assay.

The order of agonist potency in the adenylyl cyclase assay (5-CT > 5-HT > 8-OH-DPAT) was in agreement with that reported by Jasper *et al*. (1997) for the human 5-HT$_{7(b)}$ receptor. In addition, the order of agonist potency corresponded to the order of agonist affinity, although absolute functional potencies were lower than the corresponding binding affinities (Table 1). This difference, which has been noted previously (e.g. Eglen *et al*., 1997), is unlikely to be explained by poor coupling efficiency since in the present study 8-OH-DPAT was a full agonist compared to 5-HT, although 8-OH-DPAT lacks efficacy at the native human 5-HT$_7$ receptor in vascular smooth muscle cells (Schoeffter *et al*., 1996) and is a partial agonist at the rat cloned 5-HT$_7$ receptor (Lovenberg *et al*., 1993). A possible alternative explanation for the discrepancy between agonist functional potency and binding affinity is that [^{3}H]-5-CT labels a different agonist affinity state of the receptor compared to that mediating the functional response.

The profile of action of a number of antagonists was also investigated in this system. The 'classical' 5-HT receptor antagonists methiothepin, mesulergine and ketanserin antagonised surmountably the 5-CT stimulation of adenylyl cyclase activity. Risperidone, clozapine and olanzapine, which showed similar binding affinities to those reported by Roth *et al*. (1994) at the rat 5-HT$_7$ receptor, also displayed surmountable antagonist profiles. The rank order of potency for these compounds was risperidone > clozapine > olanzapine, in agreement with the rank order of 5-HT$_7$ receptor binding affinity. The antagonist potencies of both clozapine and methiothepin were similar to those reported at the 5-HT$_7$ receptor present in human vascular smooth muscle (Schoeffter *et al*., 1996). The recently reported selective 5-HT$_7$ receptor antagonist, SB-258719 (Forbes *et al*., 1998), showed a similar antagonist profile to the above-mentioned compounds, producing a concentration-related rightward-shift of the 5-CT concentration-response curve with no significant alteration in the maximal 5-CT response. Schild analysis of the data for SB-258719 was consistent with a competitive antagonist profile (Figure 3). For the antagonists tested, there was a good correlation between the order of antagonist potency (pK$_B$) and the order of binding affinity (Table 1). In addition, for each antagonist, absolute values for antagonist potency and binding affinity were comparable. In contrast to these data, Jasper *et al*. (1997) reported that for the human 5-HT$_{7(b)}$ receptor stably expressed in HEK293 cells (at an expression level similar to that described here), antagonist potencies to inhibit 5-HT-stimulated cAMP accumulation (using intact cells) were somewhat lower than antagonist affinities determined from [^{3}H]-5-CT binding assays. The reason for these differences between the present findings and those of Jasper *et al*. (1997) is unclear, but may be related to the different splice variants or to the different method (ie cAMP accumulation in intact cells versus adenylyl cyclase activity in membranes) used to determine receptor function.

In addition to their antagonist effects, these compounds also produced a concentration-related inhibition of basal adenylyl cyclase activity. The rank order of potency to inhibit basal activity (pIC$_{50}$) correlated well with both antagonist potency (pK$_B$) and binding affinity (pK$_i$) (Table 1), although for a number of the antagonists including risperidone and methiothepin, absolute pIC$_{50}$ values were slightly lower than the corresponding apparent pK$_B$ values. The reason for this difference is unclear. The correlation between antagonist potency and potency to inhibit basal activity suggests that the mechanism by which these compounds inhibit basal activity is 5-HT$_7$ receptor-mediated. In support of this, no stimulation or inhibition of basal activity (by 10 μM 5-CT or 10 μM clozapine respectively) could be detected using membranes prepared from untransfected (parental) HEK293 cells.

The high basal adenylyl cyclase activity seen in the present study is consistent with constitutive 5-HT$_7$ receptor coupling and the inhibition of basal activity is therefore consistent with inverse agonism. However, a possible alternative explanation is that the compounds are fully or partially antagonising a stimulatory effect of endogenous 5-HT, as discussed previously by Baxter and Tilford (1995). The concentration of endogenous 5-HT required to account for the present results in this way is approximately 21 nM, estimated from the data in Figure 1 and Table 1 (see Appendix). This is approximately five times higher than the concentration of 5-HT measured in the medium used to culture the cells and therefore much higher than the concentration of 5-HT likely to be carried over into the final assay. This analysis, together with the studies confirming the effectiveness of the membrane washing procedure, suggests that basal adenylyl cyclase activity reported here is unlikely to be due to stimulation of 5-HT$_{7(a)}$ receptors by endogenous 5-HT.

The mechanism of inhibition of basal adenylyl cyclase activity seen in this study is consistent with inverse agonism to reduce constitutive receptor-G-protein coupling. In relation to this, the degree of inverse agonism to inhibit basal activity varied. Risperidone, methiothepin, mesulergine, clozapine and olanzapine displayed profiles consistent with full inverse agonism, whilst both mesulergine and SB-258719 showed apparent partial inverse agonism. Constitutive receptor activity and inverse agonist effects have been reported for a variety of G-protein-coupled receptors (for review see Kenakin, 1996) including cloned 5-HT$_{1A}$ (Newman-Tancredi *et al*., 1997), 5-HT$_{1B}$, 5-HT$_{1D}$ (Thomas *et al*., 1995a, 1995b; Selkirk *et al*., 1997), 5-HT$_{2B}$ (Thomas *et al*., 1996) and (rat) 5-HT$_{2C}$ (Barker *et al*., 1994; Westphal and Sanders-Bush, 1994)

receptors. Such constitutive receptor activity has been explained in terms of a model where receptors exist in two states in the absence of agonist, an 'activated' state which promotes a functional response and an inactive state (e.g. Leff, 1995). Inverse agonists are proposed to act by binding preferentially to uncoupled receptors, thus promoting deactivation of activated receptors, which is observed as a reduction in basal activity in receptor functional assays.

Constitutive receptor activity and inverse agonism appear to be system-related phenomena dependent on a number of factors including receptor expression density and the efficiency of coupling of the receptor-G-protein-effector pathway. In relation to this, Jasper *et al*. (1997), who investigated the function of the human 5-HT$_{7(b)}$ receptor stably expressed in HEK293 cells, did not report either constitutive receptor activity or inverse agonism by compounds which display clear inverse agonist profiles in the present study (e.g. methiothepin and clozapine). Although it is impossible to rule out either system-related effects or differences in the degree of constitutive activity for the long versus the short splice variants of the human 5-HT$_7$ receptor, it is more likely that the difference is related to the different method (i.e. cAMP accumulation in intact cells) and experimental design used by Jasper *et al*. The basal rate of cAMP accumulation in their study was small and agonist stimulation gave rise to a large increase above basal so that small inhibitory effects on basal activity may have been difficult to detect. In addition, Jasper *et al*. determined the effects of antagonists on 5-HT stimulated cAMP levels but did not report effects of these antagonists on basal cAMP levels. Therefore, it is not possible to determine whether inverse agonism occurred in the study of Jasper *et al*. (1997).

To date, there have been no reports of constitutive activity for the 5-HT$_7$ receptor in native tissues. This may be due in part to the low level of 5-HT$_7$ receptor expression in native tissues compared to that for the clonal cell line used in the present study. For example, To *et al*. (1995) reported a 5-HT$_7$ receptor density of 69 fmoles mg^{-1} protein in guinea-pig cerebral cortex (as defined using [^{3}H]-5-CT binding) compared with greater than 4 pmoles/mg^{-1} protein for the cell line used in the present study.

In conclusion, in the present study we have demonstrated that the functional profiles for agonists and antagonists agree well with their binding affinities for the human cloned 5-HT$_{7(a)}$ receptor stably expressed in HEK293 cells. We have also presented evidence for constitutive receptor activity in this expression system and for inverse agonist effects for a number of antagonists including clozapine and the 5-HT$_7$ receptor-selective antagonist, SB-258719.

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Appendix

Theoretical prediction of the concentration of endogenous 5-HT required to explain observed basal adenylyl cyclase activity

Assume that the true basal adenylyl cyclase activity is that measured in the presence of high concentrations of full 'inverse agonists', e.g. clozapine or olanzapine. The data in Figure 1 can then be used to estimate the endogenous 5-HT that would have been required in the adenylyl cyclase assay to account for the observed basal activity.

Assuming 5-HT-stimulated adenylyl cyclase activity is approximated by a simple Michaelis-type equation, V, the total adenylyl cyclase activity in the presence of added 5-HT (i.e. basal + stimulated), can be defined as:

$$V = V_o + \{(V_{max} - V_o).\ ([5\text{-}HT] + [E])/(([5\text{-}HT] + [E]) + K_m)\} \quad (1)$$

where [5-HT] is the concentration of added 5-HT, [E] is the concentration of endogenous 5-HT, K_m is the concentration of total (i.e. endogenous + added) 5-HT required for half maximal stimulation of adenylyl cyclase activity, V_{max} is the maximum 5-HT-stimulated activity and V_o is the true basal activity in the absence of any 5-HT (assumed to be the activity in the presence of a high concentration of clozapine). V_c, the activity in the absence of added 5-HT (i.e. the apparent constitutive activity), is given by (1) when [5-HT] = 0:

$$V_c = V_o + \{(V_{max} - V_o).\ ([E])/(([E]) + K_m)\} \quad (2).$$

[X], the concentration of added 5-HT at which stimulated activity is half maximal, can be obtained by interpolation from the concentration-effect curve for added 5-HT and then:

$$Km = [X] + [E] \quad (3)$$

Solving for [E] from equations (2) and (3) gives

$$[E] = \{[X].\ (V_c - V_o)\}/\{(V_{max} - V_o) - 2(V_c - V_o)\} \quad (4).$$

From Figure 1, V_{max} and V_c are 48 and 22 pmoles min^{-1} mg^{-1} protein respectively. V_O is 4.4 pmoles min^{-1} mg^{-1} protein, assuming that clozapine inhibits basal activity by 80% (Table 1) so that the total activity at the K_m concentration of 5-HT is 26.2 pmoles min^{-1} mg^{-1} protein. From Figure 1, [X], the concentration of added 5-HT giving this activity, is 10 nM. Using these values, (4) predicts a value for [E] of about 21 nM 5-HT.