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The human 5-HT$_7$ serotonin receptor splice variants: constitutive activity and inverse agonist effects

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1 Using membranes from stably or transiently transfected HEK293 cells cultured in 5-HT-free medium and expressing the recombinant human 5-HT$_7$ receptor splice variants (h5-HT$_{7(a)}$, h5-HT$_{7(b)}$ and h5-HT$_{7(d)}$), we compared their abilities to constitutively activate adenylyl cyclase (AC).

2 All h5-HT$_7$ splice variants elevated basal and forskolin-stimulated AC. The basal AC activity was reduced by the 5-HT$_7$ antagonist methiothepin and this effect was blocked by mesulergine (neutral 5-HT$_7$ antagonist) indicating that the inhibitory effect of methiothepin is inverse agonism at the 5-HT$_7$ receptor.

3 Receptor density correlated poorly with constitutive AC activity in stable clonal cell lines and transiently transfected cells. Mean constitutive AC activity as a percentage of forskolin-stimulated AC was significantly higher for the h5-HT$_{7(b)}$ splice variant compared to the h5-HT$_{7(a)}$ and h5-HT$_{7(d)}$ splice variants but only in stable cell lines.

4 All eight 5-HT antagonists tested inhibited constitutive AC activity of all splice variants in a concentration-dependent manner. No differences in inverse agonist potencies (pIC_{50}) were observed between the splice variants. The rank order of potencies was in agreement and highly correlated with antagonist potencies (pK_b) determined by antagonism of 5-HT-stimulated AC activity (methiothepin > metergoline > mesulergine ≥ clozapine ≥ spiperone ≥ ritanserin > methysergide > ketanserin).

5 The efficacy of inverse agonism was not receptor level dependent and varied for several 5-HT antagonists between membrane preparations of transiently and stably transfected cells.

6 It is concluded that the h5-HT$_7$ splice variants display similar constitutive activity and inverse agonist properties.

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Abbreviations: AC, Adenylyl cyclase; 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine

Introduction

Serotonin (5-HT) mediates its diverse physiological effects through at least 14 different receptor subtypes, of which 13 belong to the G-protein-coupled or seven transmembrane-spanning receptor family (Hoyer *et al*., 1994). Defined on the basis of molecular, pharmacological and functional criteria, 5-HT receptors form seven discrete families, including three subtypes positively coupled to adenylyl cyclase (5-HT$_4$, 5-HT$_6$ and 5-HT$_7$) through G-proteins. Whereas many of the 5-HT receptors are encoded by a single exon, RNA editing (Burns *et al*., 1997) and alternative mRNA splicing of 5-HT receptor subtypes (Gerald *et al*., 1995; Heidmann *et al*., 1997) produce splice variants adding another level to receptor complexity. Three human 5-HT$_7$ receptor splice variants (h5-HT$_{7(a)}$, h5-HT$_{7(b)}$, h5-HT$_{7(d)}$) have been identified that are structurally identical except in their predicted intracellular carboxyl terminal (C-terminal) tail (Heidmann *et al*., 1997).

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Although physiological roles for 5-HT$_7$ receptors have been implicated in multiple organ systems, such as the cardiovascular system, CNS and digestive tract (Eglen *et al*., 1997; Saxena *et al*., 1998; Vanhoenacker *et al*., 2000), little is known about which splice variants are mediating these effects. In support of the diverse physiological roles, RT–PCR studies indicate that 5-HT$_7$ receptors are widely distributed and that all three splice variants can be co-localized in the same tissue (Heidmann *et al*., 1997; Krobert *et al*., 2001). Studies addressing the functional differences of the 5-HT$_7$ receptor splice variants at the cellular and molecular levels have yielded few insights into the specific physiological roles of h5-HT$_7$ receptor splice variants. A comparative characterization of the rat 5-HT$_7$ receptor splice variants revealed no differences in ligand binding affinities or AC activation (Heidmann *et al*., 1998). We recently reported that the three h5-HT$_7$ receptor splice variants share indistinguishable pharmacological profiles and similar abilities to stimulate AC in HEK293 cells (Krobert *et al*., 2001), indicating that the C-terminal tail does not influence ligand binding or receptor/G-protein coupling.

Studies of other receptor splice variants highlight the numerous possible roles for the C-terminal tail that may give

rise to functional diversity of h5-HT$_7$ receptor splice variants. For example, the C-terminal tail of diverse G-protein coupled receptors is known to influence desensitization properties (Vanetti *et al*., 1993; Negishi *et al*., 1993; Rousseau *et al*., 1996), intracellular trafficking (Bremnes *et al*., 2000), receptor-effector coupling (Journot *et al*., 1994; Vanetti *et al*., 1993; Gerald *et al*., 1995), ligand binding (Claeysen *et al*., 1999; Canton *et al*., 1996), G-protein specificity (Namba *et al*., 1993), interactions with PDZ domain containing proteins (Songyang *et al*., 1997; Hall *et al*., 1998; Daviet *et al*., 1999) and basal constitutive activity (Prezeau *et al*., 1996). For the h5-HT$_4$ receptor, with at least seven known splice variants differing only in the C-terminal tail (Bender *et al*., 2000), constitutive activity differs between the splice variants (Claeysen *et al*., 1999; 2000). The C-terminal tail appears to be critical for maintaining the 5-HT$_4$ receptor in an inactive conformation as truncation of the C-terminal tail significantly increases constitutive activity (Claeysen *et al*., 1999). Despite the lack of sequence homology in 5-HT$_7$ and 5-HT$_4$ C-terminal tails, structural similarities exist (both contain splice variants with a truncation and one with additional phosphorylation sites (Heidmann *et al*., 1998)), suggesting a similar role for the C-terminal tail of 5-HT$_7$ receptors.

To date, the constitutive activation of AC by 5-HT$_7$ receptor splice variants is poorly characterized. Thomas *et al*. (1998) have reported a concentration-dependent inhibition of basal AC activity to 5-HT antagonists in HEK293 cells expressing the h5-HT$_{7(a)}$ receptor splice variant indicating that the h5-HT$_{7(a)}$ is constitutively active. In HEK293 cells expressing the truncated h5-HT$_{7(b)}$ receptor variant, Jasper *et al*. (1997) did not observe constitutive activation or inverse agonism in response to the 5-HT antagonists reported by Thomas *et al*. (1998). In the only report comparing the three rat splice variants, no mention of the constitutive activity was reported (Heidmann *et al*., 1998) and currently the ability of the h5-HT$_{7(d)}$ splice variant to constitutively activate AC is unknown. Therefore, in the present study we investigated whether the h5-HT$_7$ receptor splice variants expressed in HEK293 cells differ in their ability to constitutively activate AC and in their response to inverse agonists.

Methods

Cell culture, 5-HT-free medium, stable and transient transfections

The three human 5-HT$_7$ receptor splice variants were cloned and stably or transiently expressed in HEK293 cells as described previously (Krobert *et al*., 2001). Cells were grown in 5-HT-free medium (UltraCULTURE™ general purpose serum-free medium (BioWhittaker, Walkersville, MD, U.S.A.), supplemented with L-glutamine (2 mM), penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}), as well as G418 (0.4 mg ml^{-1}) for stable cell lines to maintain selection pressure). To document that the medium was 5-HT free and thus avoid the possible confound of endogenous 5-HT in the current studies, 5-HT levels were measured using HPLC with electrochemical detection (Boix *et al*., 1997). 5-HT was undetectable in UltraCULTURE™ medium using HPLC with a detection limit less than 2 nM. Furthermore, 10 nM 5-HT added to UltraCULTURE™ was only detectable in the presence of ascorbic acid (0.4 mM), indicating that 5-HT is rapidly oxidized in the medium. Additionally, UltraCULTURE™ was unable to activate AC in HEK293 membranes expressing h5-HT$_7$ receptors. In the same membranes, foetal calf serum (Invitrogen, Carlsbad, CA, U.S.A.) activated AC with the same efficacy as 5-HT and a potency indicating it contained a concentration of $\sim$25 μM 5-HT.

Membrane preparation, radioligand binding and adenylyl cyclase assay

Membranes were prepared from stable HEK293 cell lines as well as transiently transfected HEK293 cells exactly as previously described (Krobert *et al*., 2001). Adenylyl cyclase assays and radioligand binding assays were conducted on the same day on the freshly prepared membranes or on thawed frozen membranes. Radioligand binding assays with [^{3}H]-5-CT or [^{3}H]-5-HT were performed as described (Krobert *et al*., 2001). The total number of specific binding sites (B_{max}) was estimated by the equation

$$B_{max} = y(1 + K_d/x)$$

where y is the specific binding of [^{3}H]-5-CT or [^{3}H]-5-HT, K_d the equilibrium dissociation constant and x the concentration of free [^{3}H]-5-CT or [^{3}H]-5-HT. Adenylyl cyclase activity was measured by determining conversion of [α-^{32}P]-ATP to [^{32}P]-cyclic AMP as described (Krobert *et al*., 2001). Note that 1 mM 3-isobutyl-1-methyl xanthine (IBMX; Sigma, St. Louis, MO, U.S.A.) was present in all the assays. The concentration of forskolin (Calbiochem, LaJolla, CA, U.S.A.) was 100 μM when present. Data representing inhibition (or activation) of basal AC were analysed by non-linear regression using Microsoft Excel with the Solver add-in and were fit to the equation

$$Y = b + (a - b)x/(c + x)$$

where a is basal AC activity, b is AC activity in the presence of a saturating concentration of antagonist (partial/neutral/inverse), c is IC_{50}, x is the concentration of antagonist.

Protein measurements

The protein concentration in the membrane preparations were measured with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, U.S.A.) using bovine serum albumin as a standard.

Statistics

Using SPSS (Chicago, IL, U.S.A.), oneway ANOVA with correction of alpha value for multiple *post-hoc* comparisons was conducted to determine any significant differences in basal AC activity.

Radiochemicals

[^{3}H]-5-HT (119 Ci mmol^{-1}), [^{3}H]-5-CT (95 Ci mmol^{-1}), [α-^{32}P]-ATP (400 Ci mmol^{-1}) and [^{3}H]-cyclic AMP (30–50 Ci mmol^{-1}) were from Amersham (Buckinghamshire, U.K.).

Drugs

5-Hydroxytryptamine hydrochloride (serotonin) was from Sigma (St. Louis, MO, U.S.A.). Methiothepin (metitepine, 1-[10,11-Dihydro-8-(methylthio)dibenzo[b,f]thepin-10-yl]-4-methylpiperazine) maleate and methysergide ([8b(S)-9,10-didehydro-N-[1-(hydroxymethyl)propyl]-1,6-dimethylergoline-8-carboxamide) maleate were from Tocris (Bristol, U.K.). Clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e][1,4]diazepine), ketanserin (3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1H,3H)-quinazolinedione) tartrate, mesulergine (N′-[(8a)-1,6-dimethylergolin-8-yl]-N,N-dimethylsulphamide) hydrochloride, metergoline ([[(8b)-1,6-dimethylergolin-8-yl]-methyl]carbamic acid phenylmethyl ester), ritanserin (6-[2-[4-[bis(4-fluorophenyl)methylene]-1-piperidinyl]-ethyl]-7-methyl-5H-thiazolo[3,2-a]pyrimidin-5-one) and spiperone (8-[4-(4-fluorophenyl)-4-oxobutyl]-1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one) hydrochloride were from RBI (Natick, MA, U.S.A.).

Results

Expression of human 5-HT$_7$ receptor splice variants increases basal AC activity

As listed in Table 1, HEK293 cell lines stably expressing the h5-HT$_7$ receptor splice variants displayed significantly elevated basal (non-agonist stimulated) and forskolin-stimulated AC activity compared to non-transfected HEK293 cells. Basal AC activity did not differ significantly between the h5-HT$_7$ receptor splice variants if reported as pmol mg protein^{-1} min^{-1} although a trend for higher values was seen for the h5-HT$_{7(b)}$. However, if expressed as a percentage of forskolin-stimulated AC activity (Table 1), thus controlling for individual clonal differences in maximal AC activity, basal AC activity of the h5-HT$_{7(b)}$ splice variant was significantly higher than that of h5-HT$_{7(a)}$ and h5-HT$_{7(d)}$ receptor splice variants. As depicted in Figure 1, there was no tendency for basal AC activity to increase as a function of increasing receptor density in HEK293 cell lines stably expressing the h5-HT$_7$ receptor splice variants over the range of receptor densities obtained.

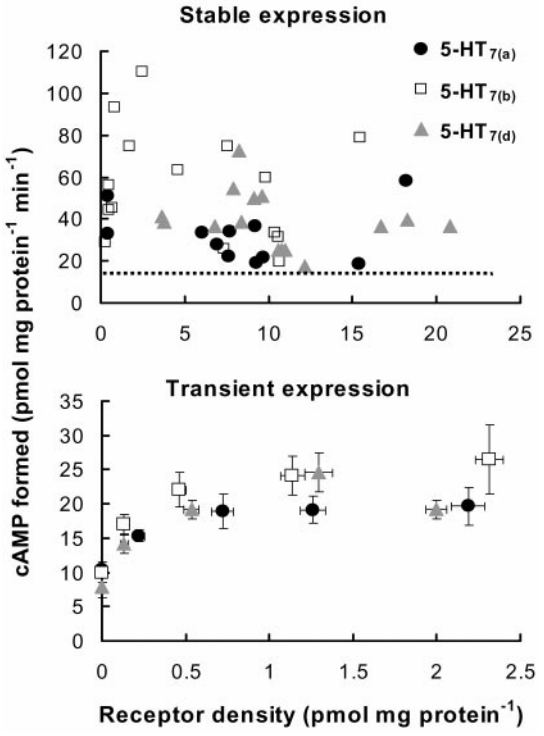

Figure 1 Basal AC activity in membranes of HEK293 cells stably (upper graph) or transiently (lower graph) expressing h5-HT$_7$ receptor splice variants plotted as a function of receptor density. Membrane AC activity was measured as described in Methods. In the upper graph, each marker represents data from one individual membrane preparation derived from a clonal HEK293 cell line stably expressing either human 5-HT$_{7(a)}$ (closed circles; $n=11$, eight clones), 5-HT$_{7(b)}$ (open squares; $n=15$, nine clones) or 5-HT$_{7(d)}$ (grey triangles; $n=15$, 10 clones) receptors. The dashed line represents the mean basal AC activity of non-transfected HEK293 cells. In the lower graph, each marker represents mean data ± s.e.mean from at least three (range 3–16) independent membrane preparations from cells with receptor expression densities within the same range.

Table 1 Constitutive adenylyl cyclase activity of human 5-HT$_7$ receptor splice variants in HEK293 cells

		Stable expression				*Transient expression*		
	HEK293 ($n=3$)	*5-HT$_{7(a)}$* ($n=11$)	*5-HT$_{7(b)}$* ($n=15$)	*5-HT$_{7(d)}$* ($n=15$)	*Mock-HEK293* ($n=17$)	*5-HT$_{7(a)}$* ($n=33$)	*5-HT$_{7(b)}$* ($n=31$)	*5-HT$_{7(d)}$* ($n=33$)
Receptor density (pmol mg protein^{-1})		8.3 ± 1.6	5.6 ± 1.3	10.5 ± 1.3		0.77 ± 0.12	0.73 ± 0.12	0.78 ± 0.11
Basal (pmol mg protein^{-1} min^{-1})	14.6 ± 0.7	32.3 ± 3.8[a]	56.0 ± 6.9[a]	39.3 ± 3.6[a]	9.1 ± 0.9	17.3 ± 0.8[d]	21.5 ± 1.3[d,e]	19.3 ± 1.0[d]
Forskolin stimulated (pmol mg protein^{-1} min^{-1})	277 ± 12	459 ± 23[b]	429 ± 34[b]	547 ± 34[b]	237 ± 12	262 ± 10	288 ± 11[f]	277 ± 11
Basal (% of max. forskolin)	5.3 ± 0.3[c]	8.2 ± 1.1[c]	13.5 ± 1.4	7.7 ± 0.5[c]	3.8 ± 0.2	6.7 ± 0.2[d]	7.4 ± 0.3[d]	6.9 ± 0.2[d]

AC activity in membranes from non-transfected HEK293 cells, mock-transfected HEK293 cells, HEK293 cells stably or transiently expressing h5-HT$_7$ receptor splice variants in the absence of agonist. Because no correlation was observed between receptor level and basal AC activity in stable clones (see Figure 1), data presented are mean ± s.e.mean of all stable clones used in the current studies. Likewise, the data are mean ± s.e.mean of all transiently transfected cells used in the current studies (data obtained from 5-HT$_{7(a)}$ – eight experiments; 5-HT$_{7(b)}$ – seven experiments; 5-HT$_{7(d)}$ – seven experiments). [a]$P<0.01$ vs HEK293, *a-priori* contrasts; [b]$P<0.05$ vs HEK293, *post-hoc* pair wise contrasts with Dunett C correction; [c]$P<0.05$ vs 5-HT$_{7(b)}$, *post-hoc* pair wise contrasts with Bonferroni adjustment of alpha values; [d]$P<0.05$ vs mock-HEK293, *a-priori* contrasts; [e]$P<0.05$ vs 5-HT$_{7(a)}$, *post-hoc* pair wise contrasts with Bonferroni adjustment of alpha values; [f]$P<0.05$ vs mock-HEK293, *post-hoc* pair wise contrasts with Bonferroni adjustment of alpha values.

Due to a paucity of clones stably expressing lower densities of h5-HT$_{7(a)}$ and h5-HT$_{7(d)}$, we transiently expressed a wider range of lower receptor densities in HEK293 cells. In contrast to the lack of receptor dependence in stable cell lines, AC activity in transiently transfected cells increased with increasing receptor density whether expressed as pmol mg protein^{-1} or as a percentage of forskolin stimulation. However, the increase appeared saturable and basal AC activity reached an asymptote at relatively low receptor density ($\sim$500 fmol mg protein^{-1}; Figure 1). Although to a lesser degree than in stable clones, HEK293 cells transiently expressing h5-HT$_7$ receptor splice variants displayed elevated basal and forskolin-stimulated AC activity (Table 1). Unlike stable cell lines, no significant difference in constitutive activation of AC was observed between the h5-HT$_7$ receptor splice variants over a wide range of receptor densities in transiently transfected HEK293 cells (Table 1). However, as in stable clones, the h5-HT$_{7(b)}$ had a tendency of higher basal AC activity compared to the h5-HT$_{7(a)}$ and h5-HT$_{7(d)}$ receptor splice variants.

Antagonists inhibit constitutive activity of human 5-HT$_7$ receptor splice variants

Basal AC activity was reduced by increasing concentrations of 5-HT$_7$ antagonists in membranes of stable cell lines expressing h5-HT$_7$ receptors (Figure 2), indicating that all three receptor splice variants display constitutive activity (activation of AC in the absence of agonist). Eight 5-HT antagonists were tested for their ability to antagonize constitutive AC activity by the three h5-HT$_7$ receptor splice variants in membranes from stably transfected HEK293 cells. As illustrated in Figure 2, similar antagonist profiles were observed for methiothepin, clozapine, ketanserin, ritanserin and spiperone across the three receptor splice variants. However, qualitative and quantitative differences were noted for metergoline, methysergide and mesulergine between the three h5-HT$_7$ receptor splice variants. As depicted, metergoline, methysergide and mesulergine only antagonized constitutive activity of the 5-HT$_{7(b)}$ splice variant. In contrast, constitutive activity of the h5-HT$_{7(a)}$ and h5-HT$_{7(d)}$ receptor splice variants was not antagonized by metergoline or mesulergine, whereas methysergide had a slight agonistic effect. The variable effect of these three antagonists was not specific to the h5-HT$_{7(a)}$ and h5-HT$_{7(d)}$ receptor splice variants since a failure to inhibit constitutive activity was observed in some h5-HT$_{7(b)}$ clones (data not shown). Likewise, metergoline, methysergide and mesulergine were partial inverse agonists at other h5-HT$_{7(a)}$ and h5-HT$_{7(d)}$ clones (data not shown). Taken together, these findings indicate that the differential responsiveness to these three antagonists was dependent on the clonal cell line and not the h5-HT$_7$ receptor splice variant expressed. In those clones where mesulergine was a neutral antagonist, the inverse

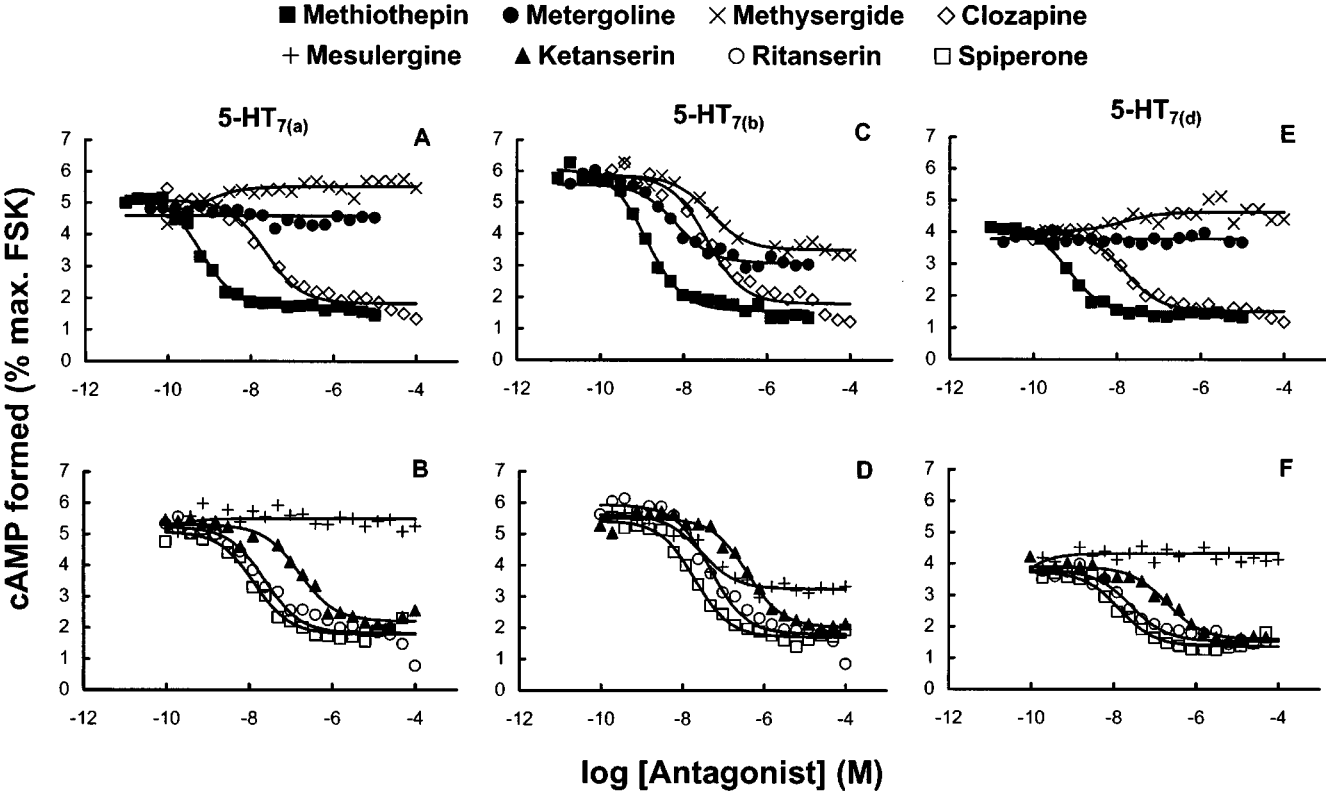

Figure 2 Effects of 5-HT receptor antagonists on basal AC activity in membranes from HEK293 cells stably expressing h5-HT$_7$ receptor splice variants at similar densities ($\sim$8–10 pmol mg protein^{-1}). Shown are two representative experiments each depicting the effect of four antagonists (A,C,E display methiothepin (solid squares), metergoline (solid circles), methysergide (solid crosses) and clozapine (open diamonds); B,D,F display mesulergine (plus signs), ketanserin (solid triangles), ritanserin (open circles) and spiperone (open squares). Membrane AC activity was measured as described in Methods in the presence of increasing concentrations of antagonist.

agonist effect of methiothepin was antagonized by increasing concentrations of mesulergine (data not shown) indicating that the antagonist inhibition of basal AC is 5-HT$_7$ receptor mediated. Interestingly, constitutive activity was revealed by inverse agonists in all clones evaluated regardless of the magnitude of elevated basal AC activity of the clone (Figure 3). As shown, the per cent reduction of basal AC activity evoked by methiothepin was equivalent between the two h5-HT$_{7(b)}$ clones ($\sim$75%) despite no significant elevation of basal AC activity of clone 1 over plain HEK293 cells.

Compared to the full inverse agonist methiothepin, clozapine, ketanserin, ritanserin and spiperone were full inverse agonists at all three h5-HT$_7$ receptor splice variants in the stable clones evaluated (Table 2). Across all stable clones tested, metergoline was a partial inverse agonist at all three h5-HT$_7$ receptor splice variants, whereas methysergide was a weak partial inverse agonist only at the h5-HT$_{7(a)}$ and h5-HT$_{7(b)}$ and mesulergine was a partial inverse agonist consistently only at h5-HT$_{7(b)}$ receptors (Table 2). Estimates of inverse agonist potencies (pIC_{50}) based on inhibition of basal AC activity in stable cell lines revealed no significant differences between the h5-HT$_7$ receptor splice variants among the eight antagonists tested (Table 2). Figure 4 compares the inverse agonist potencies estimated from these studies with the antagonist potencies determined by inhibition of 5-HT-stimulated AC (Krobert *et al*., 2001). Although a strong linear relationship was observed between these potencies, in general, inverse agonist potencies were slightly lower than antagonist potencies, primarily for the h5-HT$_{7(a)}$ and h5-HT$_{7(b)}$ receptors.

Inverse agonist efficacy varies between stably and transiently transfected HEK293 cells

In order to determine if receptor density influences inverse agonist efficacy, antagonists were tested over a range of receptor densities expressed transiently in HEK293 cells. Of the eight antagonists evaluated, receptor density did not significantly influence the efficacy of inverse agonism, although a trend of reduced inverse agonist efficacy was observed for metergoline, methysergide and mesulergine with increasing receptor density only at h5-HT$_{7(a)}$ (data not shown) and h5-HT$_{7(b)}$ (Figure 5) receptors. However, differences in inverse agonist efficacy between transiently and stably transfected HEK293 cells were observed for several antagonists (Figure 6). Only clozapine and ritanserin were full inverse agonists relative to methiothepin in both transiently and stably transfected HEK293 cells. Although ritanserin appears to be a more effective inverse agonist than methiothepin, the concentrations of ritanserin and clozapine used in these studies (100 μM) did reduce basal AC activity (26 and 18% respectively) of non-transfected HEK293 cells. Taking this non-specific activity into account, clozapine may not be a full inverse agonist, but ritanserin likely remains so at h5-HT$_7$ receptors in transiently transfected HEK293 cells. No other non-specific effects were observed for the antagonists tested at the concentrations used. Even though higher concentrations were used on transients, ketanserin and spiperone were only partial inverse agonists, whereas they were full inverse agonists in stable cell lines. Conversely, whereas metergoline, methysergide and mesulergine were weak partial inverse agonists in stable HEK293 cell lines, the inverse agonism of these three antagonists was significantly greater at all three h5-HT$_7$ receptors in transiently transfected HEK293 cells.

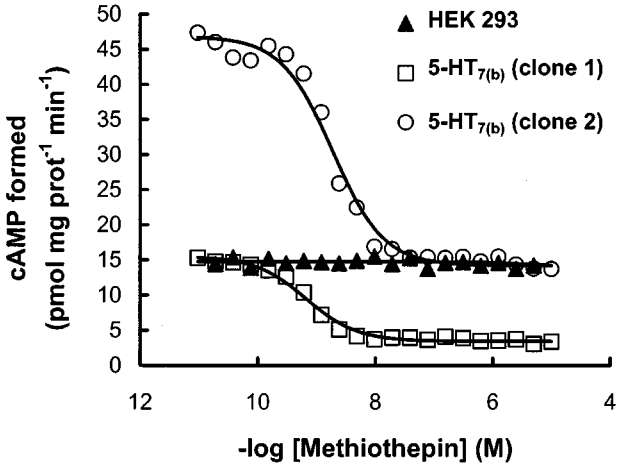

Figure 3 Effect of increasing concentrations of methiothepin on basal AC activity in membranes of non-transfected HEK293 cells and two clones stably expressing high densities of h5-HT$_{7(b)}$ receptors (10 and 15 pmol mg protein^{-1}, respectively). Note that constitutive activity of h5-HT$_7$ receptors was revealed in clone 1 even though basal AC activity was not elevated above that of non-transfected HEK293 cells. This phenomenon was also observed in clones expressing h5-HT$_{7(a)}$ and h5-HT$_{7(d)}$ receptors.

Discussion

This study provides the first comparative characterization of constitutive activity and inverse agonist properties among the three known h5-HT$_7$ receptor splice variants. As expected for constitutively active receptors, HEK293 cells stably or transiently expressing either of the h5-HT$_7$ receptor splice variants showed elevated basal AC activity over non- or mock-transfected HEK293 cells (see Table 1). Similar to Thomas *et al*. (1998) for the h5-HT$_{7(a)}$ receptor, we observed concentration-dependent inhibition of basal AC activity in response to numerous 5-HT$_7$ antagonists indicative of inverse agonism. Studies of constitutive activity and inverse agonism are often complicated by the presence of endogenous receptor activators. To eliminate such confounding effects, we used a serum-free medium and documented the absence of 5-HT by HPLC and a 'bioassay' measuring AC activation in membranes of HEK293 cells expressing 5-HT$_{7(a)}$ receptors. Furthermore, we demonstrated that a neutral 5-HT$_7$ antagonist (mesulergine) blocked methiothepin mediated reduction in basal AC activity, indicating that the inverse agonism is 5-HT$_7$ receptor mediated. Taken together, these studies indicate that all h5-HT$_7$ receptor splice variants are capable of constitutively activating AC in HEK293 cells.

In contrast, Adham *et al*. (1998) failed to observe inverse agonism in murine fibroblasts expressing h5-HT$_{7(a)}$ receptors. Although it can not be ruled out that constitutive activity of h5-HT$_7$ receptors is specific to HEK293 cells, as to date, h5-HT$_7$ receptor constitutive activity has not been reported in other cell lines, this seems unlikely. First, the 5-HT$_4$ receptor splice variants, which share structural similarities with the 5-HT$_7$ receptor splice variants (different carboxyl tails, including one

Table 2 Inverse agonist potency (pIC_{50}) and efficacy at human 5-HT$_7$ receptor splice variants determined from inhibition of constitutive adenylyl cyclase activity

	*p*IC$_{50}$			*Efficacy (% of Methiothepin)*		
	5-HT$_{7(a)}$	*5-HT$_{7(b)}$*	*5-HT$_{7(d)}$*	*5-HT$_{7(a)}$*	*5-HT$_{7(b)}$*	*5-HT$_{7(d)}$*
Methiothepin	9.04±0.11	9.07±0.11	9.18±0.09	100±8	100±6	100±4
Metergoline	7.96±0.27	8.22±0.43	8.49±0.14	26±7	36±4	20±3
Mesulergine	NA	7.73±0.25*	NA	0.0±5	47±18	−10±9
Clozapine	7.47±0.08	7.69±0.07	7.68±0.11	96±8	91±6	96±3
Spiperone	7.75±0.08	7.73±0.02	7.79±0.05	103±21	101±8	97±5
Ritanserin	7.62±0.11	7.37±0.05	7.64±0.10	110±19	100±8	93±5
Methysergide	7.36±0.37*	7.35±0.22	7.21±0.42*	18±9	19±7	−2±6
Ketanserin	6.59±0.13	6.42±0.08	6.61±0.18	106±17	95±7	93±5

AC activity was measured in the presence of increasing concentrations of antagonist in membranes of HEK293 cells stably expressing either human 5-HT$_{7(a)}$, 5-HT$_{7(b)}$ or 5-HT$_{7(d)}$ receptors, and potency and efficacy of antagonist inhibition of constitutive AC activity was determined. Potency (pIC_{50}) was calculated as described in Methods and inverse agonist efficacy is reported as a percentage of the inhibition of basal adenylyl cyclase activity produced by 10 μM methiothepin. Data are mean±s.e.mean from 3–6 individual experiments (triplicate samples) using membranes derived from at least two individual clones for each splice variant. *pIC_{50} was calculated from only two experiments due to lack of inverse agonist activity in some experiments (see Figure 2). NA: Not applicable.

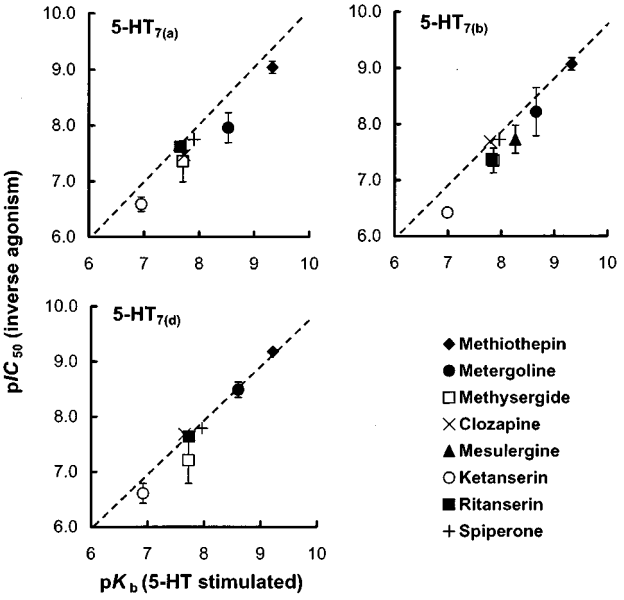

Figure 4 Relationship of inverse agonist and antagonist potencies. Comparison of pIC_{50} values obtained from antagonist-inhibition of basal AC activity (y-axis) and pK_b values calculated from antagonist-inhibition of 5-HT stimulated AC activity (x-axis) for the h5-HT$_7$ receptor splice variants. Inverse agonist pIC_{50} values were taken from Table 2 and antagonist pK_b values are from Krobert *et al.* (2001).

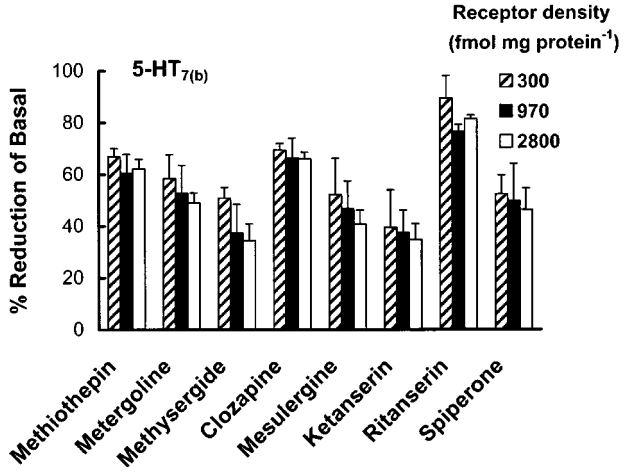

Figure 5 Effect of increasing receptor density on the efficacy of antagonists (10 μM methiothepin or metergoline; 100 μM methysergide, clozapine, mesulergine or ritanserin; 50 μM ketanserin or 33 μM spiperone) to inhibit basal AC activity in membranes of HEK293 cells transiently expressing 5-HT$_{7(b)}$ receptors. The reduction of basal was calculated as (1 minus AC activity in the presence of antagonist/AC activity in the absence of antagonist) × 100. The figure shows mean±s.e.mean of 2–4 membrane preparations with the average receptor density indicated as fmol mg protein^{-1} in the legend. Similar data were obtained for the h5-HT$_{7(a)}$ and h5-HT$_{7(d)}$ splice variants.

isoform containing additional phosphorylation sites and one truncated isoform) (Heidmann *et al.*, 1997; Blondel *et al.*, 1998; Claeysen *et al.*, 1999), display constitutive activation of AC in different cell lines (Claeysen *et al.*, 1999). Second, whereas AC enzymatic activity of membrane preparations was measured in the current studies and those of Thomas *et al.* (1998), Jasper *et al.* (1997) measured whole cell cyclic AMP accumulation in HEK293 cells expressing h5-HT$_{7(a)}$ and h5-HT$_{7(b)}$ receptors and did not detect inverse agonist effects of 5-HT antagonists. Chidiac *et al.* (1994) also observed inverse agonism for β-adrenergic antagonists only in membrane preparations and not in whole cells. Since basal AC activity is relatively low in HEK293 cells, these procedural differences likely contribute to the sensitivity of measuring constitutive AC activity and inverse agonism.

As depicted in Figure 1, clones stably expressing h5-HT$_7$ receptors had basal AC activity over non-transfected HEK293 cells, but this basal AC increase was not correlated with increasing receptor density. Although basal AC activity of clones stably expressing the h5-HT$_{7(b)}$ receptor was significantly higher (Table 1), this effect may not be specific to the h5-HT$_{7(b)}$ splice variant *per se*, since no significant difference in constitutive AC activity was seen between the splice variants in transiently transfected cells. This apparent receptor-density independence could indicate some sort of homeostatic regulation of stably expressed h5-HT$_7$ receptors or their signalling machinery. Our finding contrasts that observed for the 5-HT$_4$ receptor (Claeysen *et al.*, 1999) and other G_s-protein coupled receptors (Jackson *et al.*, 2000; Samama *et al.*, 1993; Chidiac *et al.*, 1994) where basal AC

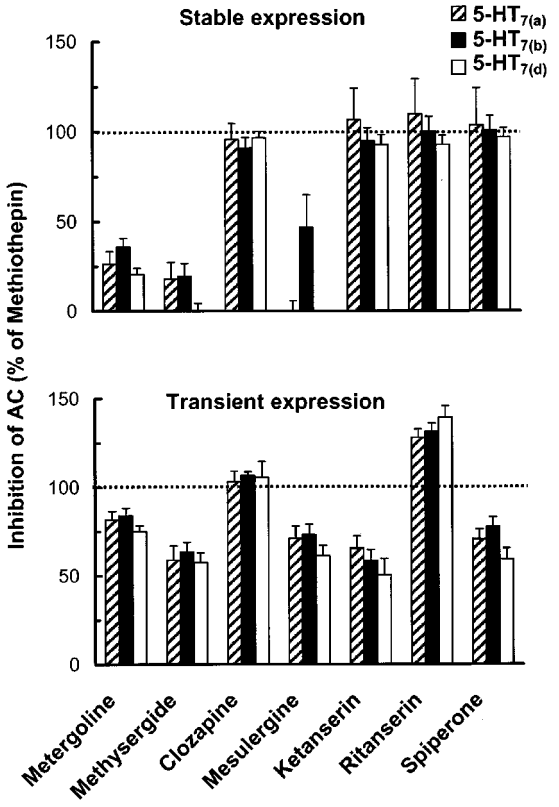

Figure 6 Efficacy of inverse agonism produced by 5-HT antagonists relative to that produced by 10 μM methiothepin (dotted line – considered a full inverse agonist) in membranes of HEK293 cells stably (top) or transiently (bottom) expressing the h5-HT$_7$ receptor splice variants. Membrane AC activity was measured as described in Methods in the presence of either 10 μM metergoline, 100 μM methysergide, clozapine, mesulergine or ritanserin, 50 μM ketanserin or 33 μM spiperone. The data shown for the transiently transfected HEK293 cells are mean $\pm$ s.e.mean of data as shown in Figure 5 collapsed across receptor density. The values shown for membranes of stable HEK293 clones are the mean $\pm$ s.e.mean obtained from the asymptote of 3–6 concentration response curves.

activity increased linearly with receptor density. However, the basal AC activity of the h5-HT$_4$ receptor reached an asymptote in LLC-PK1 cells but not in COS-7 cells at equivalent receptor density (Claeysen *et al*., 1999), indicating that different host cells may yield different results. In the present study, basal AC activity of HEK293 cells transiently expressing h5-HT$_7$ receptor splice variants showed an apparently saturable increase with increasing receptor density and reached asymptotic levels at relatively low average receptor density ($\sim$500 fmol mg protein^{-1}; Figure 1), suggesting that Gα_s and/or AC levels may be limiting in this host cell. It would be interesting to determine if increasing expression of Gα_s and or AC will modify constitutive AC activation by h5-HT$_7$ splice variants.

The receptor/G-protein stoichiometry (Burstein *et al*., 1995; Seifert *et al*., 1999; MacEwan *et al*., 1995; Kenakin, 1997) and overexpression of AC (Stevens & Milligan, 1998) influences both agonist efficacy and basal constitutive activity, as well as the efficacy of inverse agonists (Newman-Tancredi *et al*., 2000). Basal AC activity increased when Gα_s was overexpressed, even though the level of Gα_s was not rate limiting (Mullaney *et al*., 1996), suggesting that accessibility of the available G-protein pool may be a limiting factor in AC activation. In support of this hypothesis, fusing Gα_s to the β_2-adrenergic receptor increased constitutive activity (Wenzel-Seifert *et al*., 1998). Possibly, stable expression of h5-HT$_7$ receptors may alter G-protein expression (or available pools) differentially between stable clones, accounting for the apparent receptor-density independence of basal AC activity. Additionally, desensitization mechanisms evoked by constitutive activity may also contribute to modifications of basal AC activity in stable clones as observed for the thyrotropin-releasing hormone receptor (Zaltsman *et al*., 2000).

Forskolin-stimulated AC activity was significantly increased by stable expression of h5-HT$_7$ receptor splice variants and slightly by transient expression (Table 1). Similar observations were made for the constitutively active histamine H$_2$ and TSH receptors (Alewijnse *et al*., 1997). Inhibition by the H$_2$ inverse agonist cimetidine indicated that the H$_2$ receptor enhanced the forskolin response through constitutive activity, possibly by increased density of available active Gα_s (Alewijnse *et al*., 1997). Basal AC levels correlated better with forskolin-stimulated AC activity than receptor density (data not shown), suggesting a similar effect of h5-HT$_7$ receptors, likely sensitive to inverse agonists.

All eight 5-HT antagonists evaluated exhibited some degree of inverse agonism at the three h5-HT$_7$ receptors with a rank order of potency in agreement with previously reported antagonist potency and binding affinity (Krobert *et al*., 2001). No significant differences between the h5-HT$_7$ receptor splice variants were revealed by estimates of inverse agonist potencies (pIC_{50}) based on inhibition of basal AC activity in stable cell lines. Although pIC_{50} estimates for ligands calculated from these studies of constitutive inhibition were highly correlated to pK_b values determined by inhibition of 5-HT-stimulated adenylyl cyclase (Krobert *et al*., 2001), the pIC_{50} estimates were slightly lower (Figure 4). Lower pIC_{50} estimates were also derived from inverse agonist effects at the h5-HT$_{7(a)}$ splice variant by Thomas *et al*. (1998) and may, in part, reflect spare receptors due to the high level of receptor expression ($>$4000 fmol mg protein^{-1}) in both studies.

The degree of inverse agonism varied among the antagonists tested with methiothepin, clozapine, ketanserin, ritanserin and spiperone displaying profiles consistent with full inverse agonism at all three h5-HT$_7$ receptors in the stable clones evaluated (Figures 2 and 6). However, metergoline, methysergide and mesulergine could appear as partial inverse agonists, neutral antagonists or in some cases partial agonists (only methysergide and mesulergine) at all three h5-HT$_7$ receptors, depending on the clone evaluated (Figure 2). Under certain conditions, this behaviour can be expected of partial agonists, and forms the basis for their description as protean agonists, as described by Kenakin (2001). Protean agonism reflects the ability of a ligand to behave as either a partial agonist, a neutral antagonist or a partial inverse agonist depending on the basal activity of the system (quiescent or with some level of constitutive activity). Typically, three conditions can be proposed to reveal protean agonism: (1) changing the receptor system from quiescent to constitutively active; (2) increasing the concentration of available G-protein or (3) changing the efficiency of receptor-G-protein coupling. Condition one is unlikely to explain the apparent protean behaviour of ligands in our

system, since constitutive activity was apparent in all clones tested. Therefore, variable G-protein availability or efficiency of receptor-G-protein coupling are more likely explanations. Such factors could also explain why relative inverse agonist efficacy can remain equivalent despite no elevation of basal AC activity in some clones (Figure 3).

Although increasing receptor densities had no significant effect on inverse agonist efficacy in transiently transfected HEK293 cells (Figure 5), different inverse agonist efficacies were seen for several antagonists (even though receptor density was equivalent) between transiently and stably transfected HEK293 cells (Figure 6). Although the mechanisms modifying inverse agonist efficacy in the current study remain unknown, clearly modifications occur to the receptor and/or the signalling pathways of the host cell when h5-HT$_7$ receptors are constitutively expressed in HEK293 cells. Thus, it is important to note that the state of the receptor and signalling machinery contributes to the functional characteristics of ligand action.

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