British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.01194.x
www.brjpharmacol.org

RESEARCH PAPER

Antagonist interaction with the human 5-HT$_7$ receptor mediates the rapid and potent inhibition of non-G-protein-stimulated adenylate cyclase activity: a novel GPCR effect

MT Klein and M Teitler

Albany Medical College, Center for Neuropharmacology & Neuroscience, 47 New Scotland Avenue, MC 136, Albany, NY, USA

Correspondence
M Teitler, Albany Medical College, Center for Neuropharmacology & Neuroscience, 47 New Scotland Avenue, MC 136, Albany, New York 12208, USA. E-mail: teitlem@mail.amc.edu

Keywords
Neuropharmacology; 5-HT; GPCRs; drug receptor mechanisms

Received
13 July 2010
Revised
2 November 2010
Accepted
6 December 2010

BACKGROUND AND PURPOSE

The human 5-hydroxytryptamine$_7$ (h5-HT$_7$) receptor is G$_s$-coupled and stimulates the production of the intracellular signalling molecule cAMP. Previously, we reported a novel property of the h5-HT$_7$ receptor: pseudo-irreversible antagonists irreversibly inhibit forskolin-stimulated (non-receptor-mediated) cAMP production. Herein, we sought to determine if competitive antagonists also affect forskolin-stimulated activity and if this effect is common among other G$_s$-coupled receptors.

EXPERIMENTAL APPROACH

Recombinant cell lines expressing h5-HT$_7$ receptors or other receptors of interest were briefly exposed to antagonists; cAMP production was then stimulated by forskolin and quantified by an immunocompetitive assay.

KEY RESULTS

In human embryonic kidney 293 cells stably expressing h5-HT$_7$ receptors, all competitive antagonists inhibited nearly 100% of forskolin-stimulated cAMP production. This effect was insensitive to pertussis toxin, that is, not G$_{i/o}$-mediated. Potency to inhibit forskolin-stimulated activity strongly correlated with h5-HT$_7$ binding affinity (r^2 = 0.91), indicating that the antagonists acted through h5-HT$_7$ receptors to inhibit forskolin. Potency and maximal effects of clozapine, a prototypical competitive h5-HT$_7$ antagonist, were unaffected by varying forskolin concentration. Antagonist interaction with h5-HT$_6$, human β_1, β_2, and β_3 adrenoceptors did not inhibit forskolin's activity.

CONCLUSIONS AND IMPLICATIONS

The inhibition of adenylate cyclase, as measured by forskolin's activity, is an underlying property of antagonist interaction with h5-HT$_7$ receptors; however, this is not a common property of other G$_s$-coupled receptors. This phenomenon may be involved in the roles played by h5-HT$_7$ receptors in human physiology. Development of h5-HT$_7$ antagonists that do not elicit this effect would aid in the elucidation of its mechanisms and shed light on its possible physiological relevance.

Abbreviations

5-HT, 5-hydroxytryptamine; AC, adenylate cyclase; BRL54443, 5-Hydroxy-3-(1-methylpiperidin-4-yl)-1H-indole; cAMP, cyclic-adenosine monophosphate; CHO, chinese hamster ovarian; CR, concentration ratio; GPCR, G-protein coupled receptor; GTP, Guanosine-5'-triphosphate; HEK, human embryonic kidney; IBMX, isobutylmethylxanthine; ICI118551, (±)-erythro-(S,S)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride; JNK, Jun N-terminal kinase; PTx, pertussis toxin; RNAi, RNA interference; SB269970, (2R)-1-[(3-Hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride; SRE, serum response element; TR-FRET, time-resolved fluorescence resonance energy transfer

Introduction

The 5-hydroxytryptamine$_7$ (5-HT$_7$) receptor, one of 14 mammalian 5-HT (serotonin) receptors, is a G-protein coupled receptor (GPCR) and is coupled with the stimulation of adenylate cyclase (Teitler and Herrick-Davis, 1994; Gerhardt and van Heerikhuizen, 1997; Hoyer and Martin, 1997; Raymond *et al.*, 2001; Hoyer *et al.*, 2002; Kroeze *et al.*, 2002). The human 5-HT$_7$ (h5-HT$_7$) receptor is expressed in various human tissues, particularly in the frontal cortex, hypothalamus, thalamus, the lower gastrointestinal tract and in various critical blood vessels including the coronary artery, basilar artery and elsewhere in the cerebral vasculature (Bard *et al.*, 1993; Shen *et al.*, 1993; Hedlund and Sutcliffe, 2004). The h5-HT$_7$ receptor has received much attention as a drug target for the treatment of a variety of clinical disorders including psychosis, depression, migraine and sleep disorders (Bard *et al.*, 1993; Lovenberg *et al.*, 1993; Shen *et al.*, 1993; Terron, 1997; Hedlund and Sutcliffe, 2004; Abbas *et al.*, 2009).

Recent reports have indicated that the h5-HT$_7$ receptor possesses several unusual properties *in vitro*. These include a 'tight association' with G-protein (Andressen *et al.*, 2006), constitutive activity (Krobert and Levy, 2002), insensitivity to receptor reserve effects (Bruheim *et al.*, 2003) and, in violation of the ternary complex model of drug action, this receptor lacks an agonist low affinity state (Adham *et al.*, 1998; Purohit *et al.*, 2005). Of profound significance is the finding that the h5-HT$_7$ receptor is capable of binding several antagonists in a wash-resistant, pseudo-irreversible manner, rendering the receptor insensitive to agonists (Smith *et al.*, 2006). These antagonists, termed 'inactivating antagonist' are risperidone, 9-OH-risperidone, methiothepin, bromocryptine, metergoline and lisuride (Knight *et al.*, 2009). Evidence that these inactivating antagonists pseudo-irreversibly bind the receptor, rather than induce a receptor conformation that persists long after drug dissociation, comes from the wash-resistant binding of [^{3}H]-risperidone. Inactivation of the h5-HT$_7$ receptor was measured in human embryonic kidney (HEK293) cells that stably express the receptor: cells were exposed to inactivating antagonists at concentrations only 10-fold over K_i, followed by a thorough drug washout procedure and exposure to 10 μmol·L^{-1} 5-HT (a concentration 200-fold over its EC$_{50}$); 5-HT-stimulated adenylate cyclase activity was shown to be reduced to basal or near basal levels (Smith *et al.*, 2006; Knight *et al.*, 2009). Further studies revealed that inactivating antagonists also render these h5-HT$_7$-expressing cells insensitive to forskolin (Toohey *et al.*, 2009), a diterpene stimulant of cAMP production that acts directly on all isoforms of adenylate cyclase except AC9 (Stengel *et al.*, 1982). Pertussis toxin (PTx), an inhibitor of G$_{i/o}$ proteins (Katada and Ui, 1982; Katada *et al.*, 1982), could not block this effect, indicating that activation of G$_{i/o}$, the typical pathway associated with the inhibition of adenylate cyclase activity, is not involved in this novel effect on forskolin's activity. These findings demonstrated that inactivating antagonists are able to induce a conformation of the h5-HT$_7$ receptor, capable of both pseudo-irreversibly binding antagonist and able to inhibit forskolin-stimulated adenylate cyclase activity. Most antagonists did not inactivate the h5-HT$_7$ receptor, and none of these non-inactivating antagonists, presumed to be competitive antagonists, affected forskolin-stimulated activity in this experimental design. Thus, it was concluded that the pseudo-irreversible actions of the inactivating antagonists were mechanistically related to the inhibition of forskolin-stimulated adenylate cyclase activity. This implied that only inactivating antagonists could produce an inhibition of forskolin-stimulated adenylate cyclase activity.

Herein, we present a detailed characterization of the inhibition of forskolin-stimulated adenylate cyclase activity mediated by the h5-HT$_7$ receptor and unambiguously demonstrate that both inactivating and non-inactivating antagonists evoke this response. Our findings reveal that the ability to induce the inhibition of forskolin-stimulated activity is not, as previously thought, concomitant with receptor inactivation. In the preceding study (Toohey *et al.*, 2009) only the inactivating antagonists were found to inhibit forskolin-stimulated activity; however, this study employed a drug washout procedure designed to remove all but the pseudo-irreversibly binding antagonists from the cells prior to the cells being stimulated with forskolin. We speculated that if all antagonists induce this response and the effects were reversible within the time frame of the drug washout procedure (30 min), then the washout procedure would have removed only the non-inactivating antagonists from the cellular media and the effects of these drugs would have gone unobserved in this experimental design. Supporting this hypothesis, adenylate cyclase expression levels were found to be unaffected by exposing these cells to the inactivating antagonists risperidone and methiothepin and the non-inactivating antagonist clozapine, suggesting that antagonists can elicit their affects on forskolin's activity without irreversibly altering the systems required for cAMP production. To assess the possibility that a much wider field of antagonists may induce the inhibition of forskolin-stimulated activity, we exposed h5-HT$_7$-expressing cells to antagonists, omitting the drug washout procedure, and stimulated cells with forskolin. Indeed, all antagonists proved to be highly efficacious in mediating the inhibition of forskolin-stimulated activity. We present data describing this novel affect on forskolin-stimulated activity as a fundamental property of antagonist interaction with at least two of the h5-HT$_7$ receptor splice variants. We also demonstrate that β adrenoceptors and another G$_s$-coupled 5-HT receptor do not exhibit this extraordinary property, suggesting that this effect may be a unique property of the h5-HT$_7$ receptor.

Methods

Drugs

Amoxapine, BRL54443, clozapine, cyproheptadine, fluphenazine, forskolin, 5-hydroxytryptamine, isobutylmethylxanthine, (±)-erythro-(S,S)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride, ketanserin, metergoline, methiothepin, metoprolol and (2R)-1-[(3-Hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride (SB269970) were provided by Sigma-Aldrich/RBI, St. Louis, MO, USA. Bromocryptine, mesulergine and methysergide were provided by Sandoz Laboratories, Basel, Switzerland. Risperidone and

9-OH-risperidone were provided by Fitzgerald Industries International, Inc./RDI, Acton, MA, USA. Betaxolol was provided by Alcon, Inc., Hünenberg, Switzerland. Bisoprolol was provided by Merck & Co., Inc., Whitehouse Station, NJ, USA. Lisuride was provided by Dr Bryan Roth, University of North Carolina, Chapel Hill.

Cell culture

Several recombinant cell lines were cultured that heterologously express GPCRs; the names we use to identify these receptor types conform to the British Journal of Pharmacology's Guide to Receptors and Channels (Alexander *et al.*, 2009). HEK293 cells stably expressing the h5-HT$_{7(a)}$ receptor splice variant were cultured as previously described (Toohey *et al.*, 2009) in minimal essential media (MEM; Mediatech, Inc., Manassas, VA, USA) containing 10% foetal bovine serum (FBS; Thermo Scientific HyClone, South Logan, UT, USA), 1% penicillin/streptomycin cocktail (Gibco/Invitrogen Corp., Carlsbad, CA, USA) and 0.2 mg·mL^{-1} G418 (Gibco). These cells express h5-HT$_7$ receptors at a density of 2.8 pmol·mg^{-1} of protein (Knight *et al.*, 2009). CHO-K1 cells stably expressing the h5-ht$_{1E}$ receptor were generated by Scripps Research Institute (Scripps Research Molecular Screening Center, Jupiter, FL, USA) and cultured in DMEM (Dulbecco's modified Eagle's medium; Mediatech) containing 10% FBS, 1% penicillin/streptomycin/neomycin cocktail (Gibco), 0.1 mmol·L^{-1} non-essential amino acids (Gibco), 25 mmol·L^{-1} HEPES (Gibco), and 1 mg·mL^{-1} G418. CHO-K1 cells stably expressing the h5-HT$_6$ receptor were generated previously (Purohit *et al.*, 2005) and were cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin and 0.2 mg·mL^{-1} G418. The following cell lines were donated by Dr Bryan Roth, University of North Carolina, Chapel Hill, NC, USA: HEK293 cells that stably express the h5-HT$_{7(a)}$ receptor splice-variant were cultured in MEM containing 10% FBS and 0.1 mg·mL^{-1} gentamicin. HEK293 cells that stably express the h5-HT$_{7(b)}$ receptor splice-variant were cultured in DMEM containing 10% FBS and 2 μg·mL^{-1} puromycin. CHO-K1 cells stably expressing the human β_1 adrenoceptor and HEK293 cells stable expressing either the human β_2 or human β_3 adrenoceptors were cultured in DMEM/F-12 containing 10% FBS and 0.1 mg·mL^{-1} hygromycin B.

cAMP accumulation assay

Total cAMP was measured using the LANCE cAMP detection kit (PerkinElmer, Inc., Waltham, MA, USA) according to the manufacture's directions with modifications. All cells were cultured in 100 mm dishes for 18 h in serum-free media prior to assay. For some experiments, cells were cultured with 100 ng·mL^{-1} PTx (Sigma-Aldrich) in parallel with untreated cells in serum-free media for 18 h (Katada *et al.*, 1982). Cells were lifted using 1 mL per dish Versene (Invitrogen), followed by the addition of 11 mL per dish phosphate-buffered saline and centrifugation for 5 min at 330× *g*. The supernatant was aspirated, the cell pellet was resuspended in stimulation buffer (Hank's balanced salt solution containing 5 mmol·L^{-1} HEPES, 0.5 mmol·L^{-1} isobutylmethylxanthine and 0.1% bovine serum albumin), and cells were counted in a haemacytometer and diluted as necessary. Cells were pre-incubated with antagonists for 30 min at room temperature prior to exposure to agonist or forskolin; 5 μL of pre-incubated cells were added to a 384-well white opaque microtitre plate, in which wells contained 5 μL of stimulation buffer containing: agonist or forskolin at twice the final concentration, a concentration of antagonist equal to the pre-incubation concentration and the LANCE anti-cAMP antibody at a 1:100 dilution. Cells were incubated for 30 min at room temperature; the reaction was stopped and cells were lysed by the addition of 10 μL detection buffer (prepared according to the PerkinElmer LANCE cAMP detection kit manual). The assay plate was incubated for another 2 h at room temperature, then time-resolved fluorescence resonance energy transfer (TR-FRET) was detected by a Victor3 1420 multilabel plate-reader (PerkinElmer) using the settings defined by the LANCE cAMP detection kit manual. To determine maximal forskolin activity, receptor responsiveness and basal activity, cells pre-incubated in the absence of drug were added to wells containing only forskolin, agonist and stimulation buffer respectively.

Data analysis

Total cAMP accumulated was determined by interpolation of TR-FRET values on a cAMP standard curve best-fit to a one-site competition curve. Percent maximal forskolin activity was determined by subtracting basal cAMP accumulation from all values, then normalizing values to cAMP accumulated by cells exposed to only forskolin (no drug treatment). Nonlinear regression analysis of concentration–response data and all other statistical analyses were performed by Prism 5.0 (GraphPad Software, San Diego, CA, USA). Drug potency was expressed as either pEC$_{50}$ (–log EC$_{50}$, mol·L^{-1}) or pIC$_{50}$ (–log IC$_{50}$, mol·L^{-1}). Schild analysis (Arunlakshana and Schild, 1959) of clozapine antagonism of 5-HT concentration-responses was performed globally on all data sets to obtain a pA$_2$ value for clozapine (–log K_B, mol·L^{-1}). The concentration-ratio was calculated by dividing the EC$_{50}$ of 5-HT in the presence of antagonist by the EC$_{50}$ of 5-HT in the absence of antagonist.

Results

Fourteen antagonists, all of which have been previously described as either neutral antagonists or inverse agonists at the h5-HT$_7$ receptor (Thomas *et al.*, 1998; Krobert and Levy, 2002; Purohit *et al.*, 2005), were screened for their ability to inhibit forskolin-stimulated adenylate cyclase activity in HEK293 cells stably expressing h5-HT$_7$ receptors (Figure 1). The cell line and techniques used for measuring cAMP accumulation are identical to those used in the previous study (Toohey *et al.*, 2009), except that antagonists were not removed from the cellular media prior to forskolin stimulation (see *Methods*). All antagonists, both non-inactivating and inactivating antagonists, were able to inhibit most, if not all, forskolin-stimulated activity; exposure to agonist produced an additive response to forskolin (data not shown). In order to determine the contribution of G$_{i/o}$ activity to the inhibition of forskolin-stimulated adenylate cyclase activity, cells were incubated with and without 100 ng·mL^{-1} PTx for 18 h prior to the assay. No significant differences were observed between PTx-treated and untreated cells, precluding the pos-

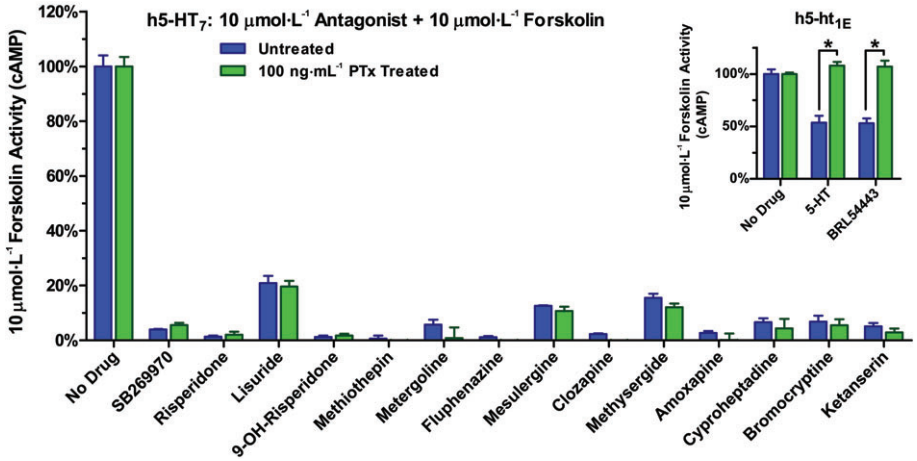

Figure 1

5-hydroxytryptamine (5-HT) antagonists inhibit forskolin-stimulated cAMP production in human embryonic kidney 293 (HEK293) cells that stably express human 5-HT_7 (h5-HT_7) receptors. Cells were cultured for 18 h in serum-free media with and without 100 ng·mL^{-1} pertussis toxin (PTx). Cells were exposed to 10 μmol·L^{-1} concentrations of antagonist for 30 min, and then exposed to 10 μmol·L^{-1} forskolin in the presence of antagonist (see *Methods*). All antagonists significantly inhibited forskolin-stimulated activity ($P < 0.0001$, one-way ANOVA with Dunnett's post test). PTx treatment did not significantly alter the effects of antagonists (two-way ANOVA with Bonferroni post tests), indicating that the inhibition of forskolin activity is not mediated by $G_{i/o}$. Inset**:** as a positive control for PTx activity, 10 μmol·L^{-1} forskolin activity was assessed in PTx treated and untreated cells stably expressing the G_i-coupled h5-ht_{1E} receptor; inhibition of 10 μmol·L^{-1} forskolin by 10 μmol·L^{-1} agonist (5-HT, BRL54443) was blocked by PTx treatment (*$P < 0.0001$, two-way ANOVA with Bonferroni post tests). Data are normalized to 10 μmol·L^{-1} forskolin-stimulated activity in the absence of drug and are the means ± SEM of three independent experiments performed in triplicate.

sibility of antagonist-induced activation of $G_{i/o}$. PTx treatment did not significantly alter basal adenylate cyclase activity, nor did it affect the cells' sensitivity to 5-HT or forskolin (Supporting Information Figure S1A). As a positive control for PTx activity, cells stably expressing the h5-ht_{1E} receptor, a G_i-coupled receptor (McAllister *et al.*, 1992), were cultured in parallel with the h5-HT_7-expressing cells in the presence and absence of 100 ng·mL^{-1} PTx, and were assayed for the inhibition of 10 μmol·L^{-1} forskolin activity (Toohey *et al.*, 2009).

Both non-inactivating and inactivating antagonists produced a robust inhibition of forskolin-stimulated activity in Figure 1, yet only inactivating antagonists inhibited forskolin's activity in the previous study (Toohey *et al.*, 2009). To explain this discrepancy, we propose that the binding of non-inactivating antagonists, unlike the inactivating antagonists, is reversible at the h5-HT_7 receptor, allowing these antagonists to dissociate from the h5-HT_7 receptor during the drug washout procedure of the previous study prior to the application of forskolin. The reversible binding nature of the non-inactivating antagonists can be confirmed by observing the competitive inhibition of agonist stimulation of the 5-HT_7 receptor, that is, Schild analysis. Clozapine, a non-inactivating antagonist, is a typical antagonist of the h5-HT_7 receptor and is assumed to be competitive with 5-HT, yet no previous reports exist describing clozapine's activity at h5-HT_7 receptors expressed in suspended cells. Figure 2 demonstrates the competitive nature of clozapine's inhibition of 5-HT activity at the h5-HT_7 receptor in our assay system. Schild analysis reveals a pA_2 value for clozapine of 7.35 ± 0.13 (K_B = 45 nmol·L^{-1}), which corresponds to the pK_i of clozapine at the h5-HT_7 receptor, 7.40 (Purohit *et al.*, 2005). The slope

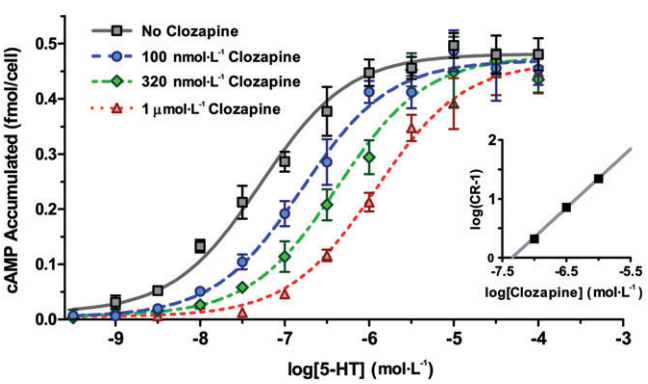

Figure 2

Clozapine is a competitive antagonist at human 5-hydroxytryptamine$_7$ (h5-HT_7) receptors expressed in human embryonic kidney 293 (HEK293) cell suspensions. Cells were pre-incubated with buffer or 100 nmol·L^{-1}, 320 nmol·L^{-1} or 1 μmol·L^{-1} clozapine for 30 min. This was followed by a 30 min incubation with 5-HT at concentrations ranging from $10^{-9.5}$ to 10^{-4} mol·L^{-1} in the presence of pre-incubation levels of clozapine. Clozapine's blockade of 5-HT-stimulated cAMP production is fully surmountable; there is no significant difference in maximal 5-HT activity ($P = 0.907$, *F*-test). pEC_{50} was determined to be 7.28 ± 0.10 for 5-HT in the absence of clozapine and 6.79 ± 0.09, 6.37 ± 0.09, and 5.92 ± 0.10 in the presence of 100 nmol·L^{-1}, 320 nmol·L^{-1}, and 1 μmol·L^{-1} clozapine respectively. Inset**:** global Schild analysis of these data determined a slope of 1.02 ± 0.18 and a pA_2 value for clozapine of 7.35 ± 0.13, which matches clozapine's affinity for the h5-HT_7 receptor (pK_i = 7.40). See *Methods* for Schild analysis description. Data are the means ± SEM of three independent experiments performed in triplicate.

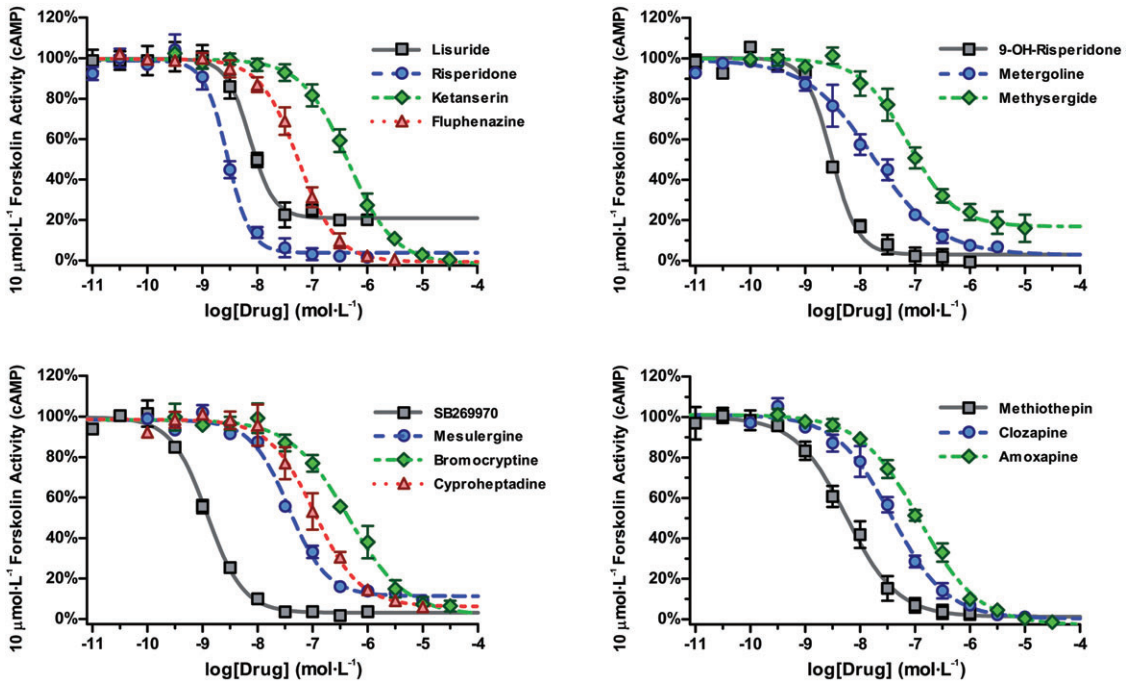

Figure 3

Concentration-responses for the inhibition of 10 µmol·L^{-1} forskolin activity in human embryonic kidney 293 cells stably expressing human 5-hydroxytryptamine$_7$ receptors. Cells were exposed to varying concentrations of antagonist and assayed for 10 µmol·L^{-1} forskolin-stimulated cAMP production as described in Figure 1. Sigmoidal concentration–response curves were best-fit by nonlinear regression. Data sets are grouped for clarity and are in no particular order. Data are normalized to 10 µmol·L^{-1} forskolin activity in the absence of drug and are the means ± SEM of three independent experiments performed in triplicate.

of the Schild linear regression and the hill slopes of concentration–response curves were not significantly different from unity. These findings are consistent with a competitive, reversible interaction between clozapine and the h5-HT$_7$ orthosteric binding site.

Concentration-responses were conducted for the inhibition of 10 µmol·L^{-1} forskolin-stimulated activity by h5-HT$_7$ receptor antagonists (Figure 3). Cell treatments were identical to the procedures used in Figure 1. Data were normalized to cAMP accumulated in the presence of 10 µmol·L^{-1} forskolin alone and were fit to sigmoidal concentration–response curves, generating pIC$_{50}$ values reflecting drug potency for the inhibition of 10 µmol·L^{-1} forskolin activity (Table 1). As seen in Figure 4, these potencies highly correlate with drug affinities for the h5-HT$_7$ receptor (r^2 = 0.91). These results suggest that antagonists act specifically through the h5-HT$_7$ receptor to inhibit forskolin-stimulated adenylate cyclase activity, and that this response is not subject to the effects of receptor reserve (see *Discussion*). Most drugs reduced forskolin-stimulated activity to levels indistinguishable from basal, while others appear to be less than 100% effective. This may be indicative of a continuum of intrinsic efficacies that h5-HT$_7$ receptor antagonists possess for the inhibition of forskolin, analogous to partial and full agonism. The maximal effects of antagonists determined by concentration–response curves in Figure 3 were consistent with the effects presented in Figure 1.

Figure 5 demonstrates that the mechanism of inhibition of forskolin-stimulated activity is non-competitive with forskolin and is insurmountable. Representative of other h5-HT$_7$ antagonists, clozapine's maximal effects and potency were unaltered by varying the concentration of forskolin used to stimulate the production of cAMP in the HEK293 cells expressing the h5-HT$_7$ receptor. This is further evidence that the site of action of these antagonists is not the forskolin binding site. These results also indicate that the inhibition of forskolin-stimulated activity, evoked by both inactivating or non-inactivating antagonists, is insurmountable and non-competitive with forskolin, which has significant implications for the possible nature of the interaction between adenylate cyclase and the effector mediating the inhibition of forskolin's activity (see *Discussion*).

Thus far, all the data presented were produced using a single recombinant cell line stably transfected to express the h5-HT$_{7(a)}$ splice variant, the full-length isoform of the receptor (Heidmann *et al.*, 1997). We sought to determine if a unique circumstance has arisen in these cells that has allowed for the h5-HT$_7$ receptor to inhibit forskolin activity and if a different splice variant of the h5-HT$_7$ receptor could similarly mediate this unprecedented effect on forskolin's activity. To achieve this, two additional HEK293 cell lines were obtained, one of which stably expresses the h5-HT$_{7(a)}$ receptor, and was generated independently from the h5-HT$_7$-expressing cell line employed elsewhere in this study. The second cell line stably expresses the h5-HT$_{7(b)}$ splice variant, the carboxy-terminal truncated isoform of the receptor. As demonstrated in Figure 6, the inhibition of forskolin-stimulated activity by h5-HT$_7$ receptors is not limited to a single stable cell line or a

Table 1

Drug binding affinities at the h5-HT$_7$ receptor and potency for the inhibition of forskolin-stimulated activity

Drug	h5-HT$_7$ affinity (pK$_i$)	Potency to inhibit forskolin (pIC$_{50}$)
1. SB269970	8.90[a]	8.91 ± 0.04
2. Risperidone	8.75[b]	8.54 ± 0.04
3. Lisuride	8.66[c]	8.13 ± 0.05
4. 9-OH-Risperidone	8.57[c]	8.52 ± 0.04
5. Methiothepin	8.43[d]	8.25 ± 0.08
6. Metergoline	8.19[d]	7.76 ± 0.10
7. Fluphenazine	7.89[b]	7.25 ± 0.05
8. Mesulergine	7.75[d]	7.43 ± 0.04
9. Clozapine	7.40[b]	7.42 ± 0.06
10. Methysergide	7.08[d]	7.13 ± 0.08
11. Amoxapine	7.39[e]	6.92 ± 0.05
12. Cyproheptadine	6.91[d]	6.97 ± 0.07
13. Bromocryptine	6.85[c]	6.35 ± 0.09
14. Ketanserin	6.33[f]	6.35 ± 0.05

Affinities are presented as pK$_i$ (-log K_i, mol·L^{-1}) and are from [a]Lovell *et al.*, 2000; [b]Purohit *et al.*, 2005; [c]Smith *et al.*, 2006; [d]Bard *et al.*, 1993; [e]Roth *et al.*, 1994; and [f]Krobert *et al.*, 2001. Potency for the inhibition of 10 µmol·L^{-1} forskolin activity in HEK293 cells stably expressing h5-HT$_7$ receptors is presented as pIC$_{50}$ (–log IC$_{50}$, mol·L^{-1}) and are the means ± SEM of three independent experiments performed in triplicate (Figure 3).

h5-HT7, human 5-hydroxytryptamine7; SB269970, (2R)-1-[(3-Hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride.

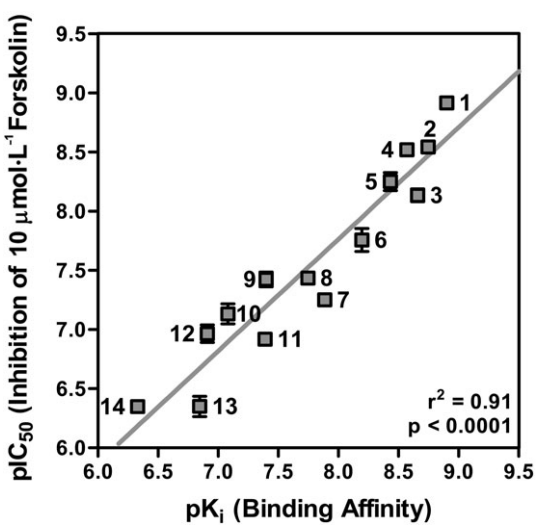

Figure 4

Antagonists act through the human 5-hydroxytryptamine$_7$ receptor to evoke the inhibition of forskolin-stimulated activity. Potencies (pIC$_{50}$) for the inhibition of 10 µmol·L^{-1} forskolin-stimulated activity, determined by concentration-responses (Figure 3), are plotted for each antagonist as a function of binding affinity (pK$_i$) at the h5-HT$_7$ receptor (r^2 = 0.91, P < 0.0001). Linear regression gives a slope of 0.95 ± 0.05 and y-intercept of 0.20 ± 0.40. Numbered points correspond to drugs listed in Table 1 and are the means ± SEM.

particular receptor splice variant. Forskolin-stimulated activity of both cell lines was sensitive to 10 µmol·L^{-1} of the antagonist and is a concentration-dependent phenomenon, as represented by clozapine concentration–response curves. Two-way ANOVA did not reveal a significant interaction between the maximal effects of drugs at the h5-HT$_{7(a)}$ and h5-HT$_{7(b)}$ receptors, meaning that the relative efficacies of drugs were not different. The pIC$_{50}$ values for clozapine to inhibit 10 µmol L^{-1} forskolin-induced activity were determined to be 7.31 ± 0.06 and 7.50 ± 0.10 for the 5-HT$_{7(a)}$ and 5-HT$_{7(b)}$ receptors respectively. These potencies do not significantly differ and correspond to the affinity of clozapine at the h5-HT$_7$ receptor. Adenylate cyclase activity was stimulated in both cell lines by 10 µmol·L^{-1} 5-HT, indicating the presence of functional G$_s$-coupled 5-HT receptors; basal adenylate cyclase activity was insignificant (data not shown). It is noteworthy that characterization of these novel h5-HT$_7$ receptor-mediated effects on forskolin activity took place in stably transfected HEK293 cells. It would be of interest for future investigations to characterize these phenomena in different parental cell lines. Studies utilizing a primary cell culture endogenously expressing 5-HT$_7$ receptors would also be of great interest (Teitler *et al.*, 2010).

It has been previously reported that antagonists of G$_s$-coupled receptors do not inhibit forskolin-stimulated adenylate cyclase activity in cells or tissue expressing these receptors (Seamon *et al.*, 1981); this implies that the phenomenon described herein may be unique to h5-HT$_7$ receptors. To investigate whether antagonizing other GPCRs will affect

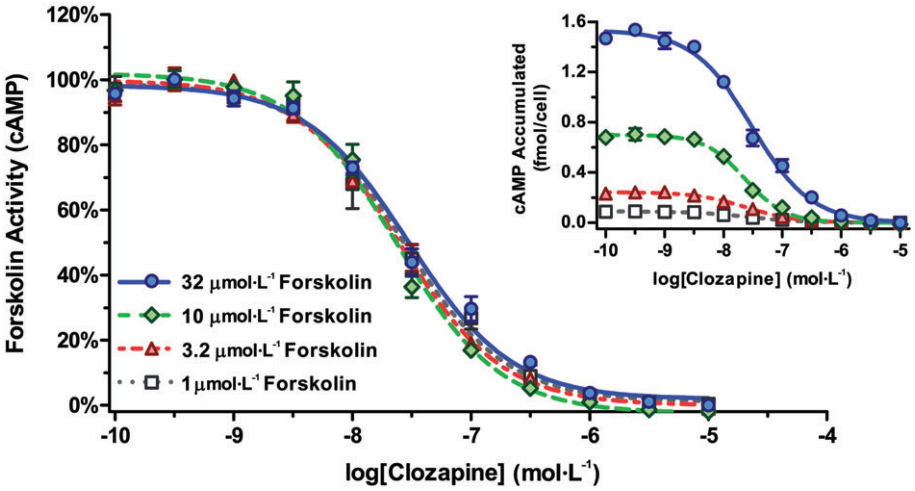

Figure 5

The human 5-hydroxytryptamine$_7$ receptor or its downstream effectors inhibit forskolin-stimulated activity in a noncompetitive manner. Cells were incubated with varying concentrations of clozapine for 30 min followed by stimulation with 1, 3.2, 10 or 32 µmol·L^{-1} forskolin in the presence of pre-incubation levels of clozapine for 30 min (see *Methods*). Clozapine pIC$_{50}$ values for the inhibition of 1, 3.2, 10 and 32 µmol·L^{-1} forskolin are 7.53 ± 0.04, 7.63 ± 0.04, 7.60 ± 0.03 and 7.57 ± 0.05, respectively, and are not significantly different from each other (P = 0.382, F test). Maximal concentrations of clozapine reduced the activity of all concentrations of forskolin to basal levels (P = 0.974, F test). Data are normalized to the cAMP produced by forskolin in the absence of clozapine. Inset: data are presented as fmol of cAMP per cell and demonstrate the concentration-dependent stimulation of adenylate cyclase by forskolin and the complete inhibition of forskolin-stimulated activity by clozapine. Data are the means ± SEM of three independent experiments performed in triplicate.

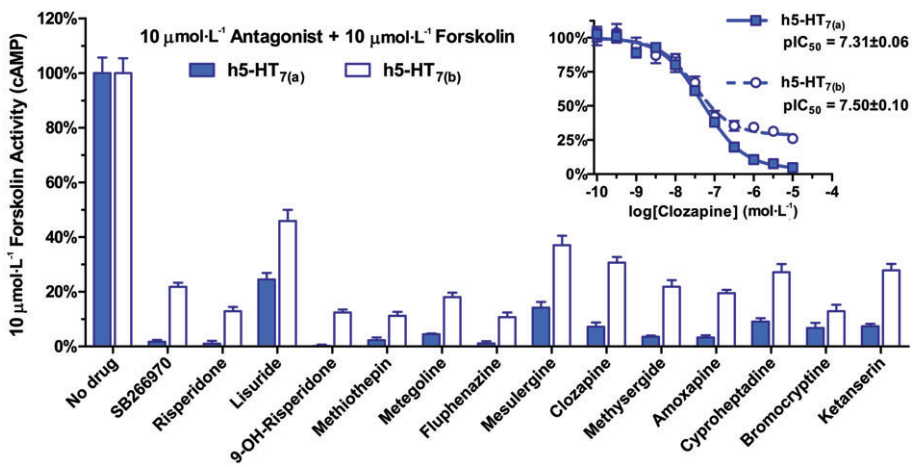

Figure 6

Human 5-hydroxytryptamine$_7$ (h5-HT$_7$) receptor-mediated inhibition of forskolin-stimulated activity is not dependent on unique stable transfections or receptor splice variant. Two new HEK293 cell lines were obtained that stably express either the h5-HT$_{7(a)}$ or h5-HT$_{7(b)}$ splice variants (see *Methods*). Inhibition of forskolin-stimulated cAMP was assayed as described in Figure 1. All antagonists significantly inhibited 10 µmol·L^{-1} forskolin activity in both cell lines (P < 0.0001, one-way ANOVA). Two-way ANOVA reveals that the maximal inhibition of forskolin activity differed between cell lines (P < 0.001), but no interaction was detected, that is, relative efficacies of drugs were identical. Exposure to 10 µmol·L^{-1} 5-HT and 10 µmol·L^{-1} forskolin, respectively, produced 0.23 and 0.61 fmol cAMP per cell in h5-HT$_{7(a)}$-expressing cells and 0.61 and 1.31 fmol cAMP per cell in h5-HT$_{7(b)}$-expressing cells. Basal activity was insignificant in both cell lines. Inset**:** clozapine-mediated inhibition of 10 µmol·L^{-1} forskolin activity is concentration-dependent in both cell lines. The pIC$_{50}$ values are not significantly different (P = 0.107, F test) and correspond to clozapine's affinity. Data are normalized to 10 µmol·L^{-1} forskolin-stimulated activity in the absence of drug and are the means ± SEM of three independent experiments performed in triplicate.

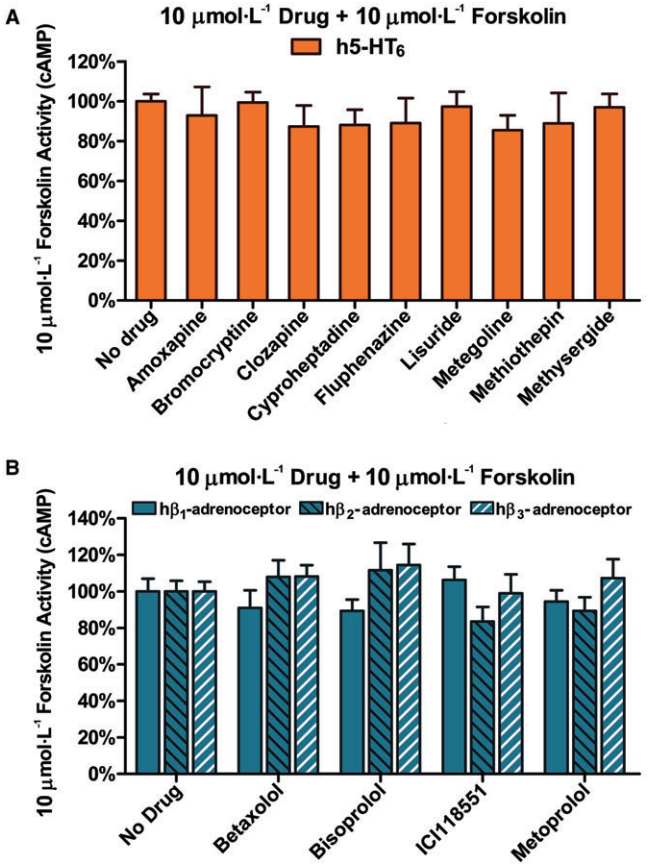

Figure 7

Antagonist interaction with the human 5-hydroxytryptamine$_6$ (h5-HT$_6$) and human β_1, β_2, and β_3 adrenoceptors does not induce the inhibition of forskolin-stimulated adenylate cyclase activity. Cells that stably express these receptors (see *Methods*) were exposed to antagonists and assayed for forskolin-stimulated cAMP production, as described in Figure 1. Data are normalized to 10 μmol·L^{-1} forskolin-stimulated activity in the absence of drug and are the means ± SEM of three independent experiments performed in triplicate. (A) 10 μmol·L^{-1} 5-HT antagonists do not inhibit 10 μmol·L^{-1} forskolin-stimulated activity in h5-HT$_6$-expressing cells (P = 0.963, one-way ANOVA); all antagonists have moderate to high affinity for the h5-HT$_6$ receptor. Exposure to 10 μmol·L^{-1} forskolin produced 0.51 fmol cAMP per cell, 10 μmol·L^{-1} 5-HT produced 0.22 fmol cAMP per cell, and basal activity was insignificant. (B) 10 μmol·L^{-1} β-adrenoceptor antagonists do not inhibit 10 μmol·L^{-1} forskolin-stimulated activity in cells stably expressing either of the human β_1, β_2, or β_3 adrenoceptors (P = 0.700, two-way ANOVA). Responsiveness to forskolin and isoprenaline varied between cell lines; in all cell lines exposure to 10 μmol·L^{-1} isoprenaline yielded at least 50% the activity of 10 μmol·L^{-1} forskolin stimulation and basal activity was insignificant.

forskolin-stimulated activity, we obtained recombinant cell lines that express the h5-HT$_6$ receptor, the human β_1, β_2 or β_3 adrenoceptors. We used drug treatment procedures identical to those used for screening antagonist effects on forskolin's activity in the h5-HT$_7$-expressing cells. As shown in Figures 7A, 10 μmol·L^{-1} concentrations of neutral antagonists and inverse agonists were unable to significantly affect forskolin-stimulated activity in cells expressing h5-HT$_6$ receptors; these antagonists all have moderate to high affinity for the h5-HT$_6$ receptor. 5-HT significantly stimulated the production of cAMP in these cells, indicating the presence of G$_s$-coupled 5-HT receptors (data not shown). As displayed in Figures 7B, 10 μmol·L^{-1} concentrations of β adrenoceptor neutral antagonists and inverse agonists with moderate to high affinity for the human β_1, β_2, and β_3 adrenoceptors were unable to inhibit 10 μmol·L^{-1} forskolin-stimulated activity in cells stably expressing these receptors. A robust stimulation of adenylate cyclase activity by isoprenaline in these cell lines indicated the presence of G$_s$-coupled β adrenoceptors (data not shown). β adrenoceptors, particularly the β_2 adrenoceptor, are considered prototypical of Class A GPCRs; the inability of β-adrenoceptor antagonists to inhibit forskolin-stimulated adenylate cyclase activity demonstrates that this novel effect of h5-HT$_7$ receptors is not a widespread phenomenon.

Discussion and conclusions

The data presented herein indicate that the h5-HT$_7$-mediated inhibition of forskolin-stimulated adenylate cyclase activity is not, as previously reported (Toohey *et al.*, 2009), restricted to the six inactivating antagonists. Indeed, the inhibition of forskolin-stimulated activity is an underlying property of all antagonist interactions with the h5-HT$_7$ receptor. In the previous study, antagonists were thoroughly removed from the cellular media prior to stimulation with forskolin. These drug washout procedures permitted Toohey *et al.* to observe the effects of only the inactivating antagonists, which pseudo-irreversibly bind to the h5-HT$_7$ receptor and, unlike competitive antagonists, would remain bound to the receptor when the cells were stimulated with forskolin. In the present study, h5-HT$_7$-expressing cells were stimulated with forskolin in the presence of an antagonist. This allowed us to detect the substantial and, in most cases, complete inhibition of forskolin-stimulated activity induced by both inactivating and non-inactivating antagonists (Figures 1, 3–5).

Published reports concerning the competitive nature of antagonist interactions with the h5-HT$_7$ receptor are limited, and among these reports different preparations of cells have yielded strikingly different and contradictory results. As a prominent example of such discrepancies, blockade of 5-HT activity at h5-HT$_7$ receptors by methiothepin was reported to be competitive and surmountable in membrane homogenate preparations (Krobert *et al.*, 2001); yet in intact cell monolayers (Smith *et al.*, 2006) and cell suspensions (Knight *et al.*, 2009), methiothepin pseudo-irreversibly binds and inactivates the h5-HT$_7$ receptor. To shed more light on the competitive nature of the non-inactivating antagonists, we scrutinized the antagonist properties of clozapine, a typical h5-HT$_7$ antagonist, in suspensions of h5-HT$_7$-expressing cells. Clozapine's inhibition of 5-HT concentration-responses in cell suspensions was fully surmountable, and the Schild analysis of these data indicates classic competitive antagonism. With this knowledge in hand, it seems likely that if clozapine and the other antagonists had been co-incubated with forskolin in the previous study, all antagonists would have significantly inhibited forskolin-stimulated activity and results would agree with those presented herein. This implies that the effects of clozapine and the other competitive antagonists on forskolin-stimulated activity were reversed

during the drug washout procedure of the previous study (30 min). This rapid recovery of forskolin's activity suggests that the mechanisms mediating the inhibition of forskolin-stimulated activity are highly dynamic and do not permanently alter the function of adenylate cyclases. This agrees with Western blot analysis of adenylate cyclase expression levels in h5-HT$_7$-expressing cells exposed to both inactivating and non-inactivating antagonists (Toohey *et al.*, 2009).

The concentration–response data and correlation of drug potencies with affinities presented in Figures 3 and 4, respectively, identifies the antagonist-evoked inhibition of forskolin activity as h5-HT$_7$ receptor-dependent. Antagonists had no effect on forskolin-stimulated activity in either parental HEK293 cells (data not shown) or in other cells that do not express h5-HT$_7$ receptors (Figure 7). These results preclude the possibility that this effect is mediated by an interaction with an endogenously expressed receptor or direct interaction with adenylate cyclase enzymes. If the inhibition of forskolin is mediated by putative biased agonist effects of these canonical antagonists, the correspondence between drug potencies and affinities demonstrates the lack of receptor reserve, where agonists would otherwise appear more potent than their affinities would predict. With no receptor reserve and the ability of h5-HT$_7$ receptors to mediate the complete inhibition of forskolin activity, these data may indicate that the ratio of h5-HT$_7$ receptors to forskolin-sensitive adenylate cyclases is very near 1:1 in these cells, and possibly a pre-existing complex between receptor and effector is present. It should be noted that antagonist (i.e. inverse agonist) effects are insensitive to receptor reserve, thus drug affinities should be predictive of potencies, making the correlation displayed in Figure 4 also supportive of the hypothesis that the effects on forskolin activity are mediated by inverse agonism.

Inverse agonism has been canonically defined as the reversal of constitutive (basal) activity of a GPCR upon binding an inverse agonist (Costa and Herz, 1989; Costa *et al.*, 1992). In order to detect inverse agonist properties of drugs, a common strategy is to generate mutant receptors that display high levels of constitutive activity (Purohit *et al.*, 2005). The use of forskolin to increase cellular cAMP levels does not induce or stabilize active conformations of the receptors; therefore, from this perspective, inhibition of forskolin's activity would not be a measure of inverse agonist efficacy. Alternatively, administration of forskolin can be seen as a means of increasing the basal adenylate cyclase activity of the cell, which may be interpreted as potentiation of a small level of constitutive receptor activity. From this perspective, the inhibition of forskolin's activity would be the inhibition of forskolin's potentiation of the h5-HT$_7$ receptor's constitutive activity; hence, the effect on forskolin's activity would be mediated by inverse agonism. This scenario, though possible, does not seem likely, and as we have demonstrated with the h5-HT$_6$ and human β adrenoceptors (Figure 7), antagonist interactions with G$_s$-coupled receptors generally do not inhibit forskolin-stimulated activity. This is consistent with 30 years of using forskolin to directly stimulate adenylate cyclase activity and was originally noted by those who discovered forskolin's effects on adenylate cyclase (Seamon *et al.*, 1981). Additionally, both inverse agonists and neutral antagonists at the h5-HT$_7$ receptor evoke the inhibition of forskolin activity. In our assay system and with our h5-HT$_7$-expressing cell line, 10 μmol·L^{-1} concentrations of cyproheptadine and bromocryptine failed to significantly inhibit basal adenylate cyclase activity and appear to be neutral antagonists (Supporting Information Figure S2A). The other 12 antagonists were found to have varying levels of inverse agonist efficacy. Thus, the inhibition of forskolin-stimulated activity is not predictive of a drug's ability to inhibit the constitutive receptor activity (r^2 = 0.008; Supporting Information Figure S2B). These observations are not consistent with inverse agonism.

The inhibition of forskolin-stimulated activity may reflect a biased agonist response, where canonical agonists (e.g. 5-HT) bind h5-HT$_7$ receptors and simulate adenylate cyclase activity through the G$_s$ pathway, and canonical antagonists stimulate other h5-HT$_7$-coupled pathways. The h5-HT$_7$ receptor has been shown to stimulate the Ca^{2+}/calmodulin-sensitive adenylate cyclases AC1 and AC8 via the elevation of intracellular Ca^{2+} levels (Baker *et al.*, 1998). However, HEK293 cells, the cell type used to characterize the h5-HT$_7$ receptor's inhibition of forskolin activity, are known to express adenylate cyclase isoforms that are not sensitive to fluctuations in Ca^{2+} concentration (Hellevuo *et al.*, 1993). The complete inhibition of forskolin-stimulated activity in HEK293 cells makes it unlikely that AC1 and AC8 are primarily responsible for this novel effect of the h5-HT$_7$ receptor. h5-HT$_7$ receptors have also been shown to signal through G$_{12}$ proteins (Kvachnina *et al.*, 2005). Stimulation of this pathway activates several downstream responses, including the activation of serum response element and Jun N-terminal kinase (Prasad *et al.*, 1995), cytoskeletal restructuring (Buhl *et al.*, 1995; Katoh *et al.*, 1998) and gene regulation via activation of Rho family GTP-ases (Fromm *et al.*, 1997). Gene regulation and other persistent effects mediated by G$_{12}$ signalling do not temporally correlate with the rapid onset and reversal of h5-HT$_7$ receptor-mediated inhibition of forskolin activity. G$_{12}$ may, however, be affecting adenylate cyclase activity in a novel manner; it would be of interest to examine this h5-HT$_7$ receptor phenomenon in cells that do not express G$_{12}$ or have low levels of G$_{12}$ activity.

The effects of h5-HT$_7$ receptor antagonists might be mediated by known adenylate cyclase inhibitory G-proteins. The inhibition of forskolin's activity is most commonly associated with G$_i$ and G$_o$ proteins. Similar to a G$_{i/o}$-mediated response, the potency and efficacy of clozapine concentration-responses were unaffected by varying the concentration of forskolin (Figure 5). This indicates a non-competitive and insurmountable interaction between adenylate cyclase and the effector(s) mediating this response; however, G$_{i/o}$ involvement is not possible. Consistent with the previous report (Toohey *et al.*, 2009), PTx, a G$_{i/o}$ inhibitor, had no effect on the ability of antagonists to inhibit forskolin-stimulated activity. There is, however, a PTx-insensitive G-protein that, similar to G$_{i/o}$, inhibits adenylate cyclase activity via direct α-subunit interaction with the enzyme. This is the G$_z$-protein (Matsuoka *et al.*, 1990; Wong *et al.*, 1992; Tso *et al.*, 2000), which is an under-characterized component of the GPCR repertoire of effectors, and may present a possible signalling pathway for the h5-HT$_7$ receptor. There are no known G$_z$-selective inhibitors, but RNAi and other genetic methods

of decreasing G_z expression or activity may be useful tools in assessing the involvement of this G-protein in the h5-HT_7 receptor's inhibition of forskolin activity.

The h5-HT_7 receptor is a well-documented G_s-coupled receptor, and involvement of this or another adenylate cyclase stimulating G-protein (G_{olf}) in this novel effect on forskolin activity cannot be categorically dismissed. It has been previously suggested that the h5-HT_7 receptor exists in a pre-complexed state with G_s, resulting in agonist potencies resistant to the effects of receptor reserve (Bruheim *et al.*, 2003). Considering this and that our results similarly show no effect of receptor reserve, this coincidence may indicate that the inhibition of forskolin-stimulated activity is mediated by G_s. As seen in other signal transduction pathways, GPCRs may engender an effector with a distinct conformation that functionally selects for a particular downstream signalling pathway (i.e. biased agonism) (Shukla *et al.*, 2008). Thus, it may be possible for antagonist-bound h5-HT_7 receptors to stimulate the accumulation of conformationally altered G_s subunits capable of associating with adenylate cyclase and non-competitively inhibiting the enzyme's responsiveness to forskolin. Thus far, no drugs have been identified that are entirely neutral at the h5-HT_7 receptor, that is, drugs that have no affect on forskolin-stimulated activity and lack the ability to stimulate adenylate cyclase. The current investigation provides a new avenue for h5-HT_7 drug development: the identification of antagonists that do not affect forskolin-stimulated adenylate cyclase activity and merely occlude the binding of other ligands (i.e. classical receptor antagonism). Such a ligand is potentially an important tool for the further characterization of this unusual 5-HT receptor; a classical receptor antagonist may help to identify unappreciated characteristics of the 5-HT_7 receptor *in vivo* and may prove to be a more advantageous therapeutic tool in the treatment of the various clinical disorders currently targeted by 5-HT_7 drug development (Abbas *et al.*, 2009). The utility of SB269970, a 5-HT_7-selective antagonist that evokes the inhibition of forskolin-stimulated activity, cannot be overlooked. *In vivo* administration of this drug in comparison to an antagonist that does not elicit this effect would be extremely useful in elucidating the physiological significance of the h5-HT_7 receptor and this highly unusual property of the receptor.

The PTx-insensitive inhibition of forskolin-stimulated adenylate cyclase activity by a GPCR is extremely rare, that the effect is mediated by a well-described G_s-coupled receptor is essentially unheard of, and that the effect is evoked by antagonist interaction with the receptor is unprecedented. The identification of this novel h5-HT_7 receptor-mediated effect in primary cells or isolated tissue preparations should provide valuable insight into how this effect on adenylate cyclase, as measured by responsiveness to forskolin, impacts endogenous cellular systems and physiological homeostasis.

Acknowledgement

This work was supported by PHS grant no. MH56650 (M.T.).

Conflicts of interest

None.

References

Abbas AI, Hedlund PB, Huang XP, Tran TB, Meltzer HY, Roth BL (2009). Amisulpride is a potent 5-HT7 antagonist: relevance for antidepressant actions in vivo. Psychopharmacology (Berl) 205: 119–128.

Adham N, Zgombick JM, Bard J, Branchek TA (1998). Functional characterization of the recombinant human 5-hydroxytryptamine7(a) receptor isoform coupled to adenylate cyclase stimulation. J Pharmacol Exp Ther 287: 508–514.

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC), 4th edition (2009). Br J Pharmacol 158 (Suppl. 1): S1–S254.

Andressen KW, Norum JH, Levy FO, Krobert KA (2006). Activation of adenylyl cyclase by endogenous G(s)-coupled receptors in human embryonic kidney 293 cells is attenuated by 5-HT(7) receptor expression. Mol Pharmacol 69: 207–215.

Arunlakshana O, Schild HO (1959). Some quantitative uses of drug antagonists. Br J Pharmacol Chemother 14: 48–58.

Baker LP, Nielsen MD, Impey S, Metcalf MA, Poser SW, Chan G *et al.* (1998). Stimulation of type 1 and type 8 Ca2+/calmodulin-sensitive adenylyl cyclases by the Gs-coupled 5-hydroxytryptamine subtype 5-HT7A receptor. J Biol Chem 273: 17469–17476.

Bard JA, Zgombick J, Adham N, Vaysse P, Branchek TA, Weinshank RL (1993). Cloning of a novel human serotonin receptor (5-HT7) positively linked to adenylate cyclase. J Biol Chem 268: 23422–23426.

Bruheim S, Krobert KA, Andressen KW, Levy FO (2003). Unaltered agonist potency upon inducible 5-HT7(a) but not 5-HT4(b) receptor expression indicates agonist-independent association of 5-HT7(a) receptor and Gs. Receptors Channels 9: 107–116.

Buhl AM, Johnson NL, Dhanasekaran N, Johnson GL (1995). G alpha 12 and G alpha 13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly. J Biol Chem 270: 24631–24634.

Costa T, Herz A (1989). Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. Proc Natl Acad Sci USA 86: 7321–7325.

Costa T, Ogino Y, Munson PJ, Onaran HO, Rodbard D (1992). Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. Mol Pharmacol 41: 549–560.

Fromm C, Coso OA, Montaner S, Xu N, Gutkind JS (1997). The small GTP-binding protein Rho links G protein-coupled receptors and Galpha12 to the serum response element and to cellular transformation. Proc Natl Acad Sci USA 94: 10098–10103.

Gerhardt CC, van Heerikhuizen H (1997). Functional characteristics of heterologously expressed 5-HT receptors. Eur J Pharmacol 334: 1–23.

Hedlund PB, Sutcliffe JG (2004). Functional, molecular and pharmacological advances in 5-HT7 receptor research. Trends Pharmacol Sci 25: 481–486.

Heidmann DE, Metcalf MA, Kohen R, Hamblin MW (1997). Four 5-hydroxytryptamine7 (5-HT7) receptor isoforms in human and rat produced by alternative splicing: species differences due to altered intron-exon organization. J Neurochem 68: 1372–1381.

Hellevuo K, Yoshimura M, Kao M, Hoffman PL, Cooper DM, Tabakoff B (1993). A novel adenylyl cyclase sequence cloned from the human erythroleukemia cell line. Biochem Biophys Res Commun 192: 311–318.

Hoyer D, Martin G (1997). 5-HT receptor classification and nomenclature: towards a harmonization with the human genome. Neuropharmacology 36: 419–428.

Hoyer D, Hannon JP, Martin GR (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav 71: 533–554.

Katada T, Ui M (1982). ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. J Biol Chem 257: 7210–7216.

Katada T, Amano T, Ui M (1982). Modulation by islet-activating protein of adenylate cyclase activity in C6 glioma cells. J Biol Chem 257: 3739–3746.

Katoh H, Aoki J, Yamaguchi Y, Kitano Y, Ichikawa A, Negishi M (1998). Constitutively active Galpha12, Galpha13, and Galphaq induce Rho-dependent neurite retraction through different signaling pathways. J Biol Chem 273: 28700–28707.

Knight JA, Smith C, Toohey N, Klein MT, Teitler M (2009). Pharmacological analysis of the novel, rapid, and potent inactivation of the human 5-Hydroxytryptamine7 receptor by risperidone, 9-OH-Risperidone, and other inactivating antagonists. Mol Pharmacol 75: 374–380.

Krobert KA, Levy FO (2002). The human 5-HT7 serotonin receptor splice variants: constitutive activity and inverse agonist effects. Br J Pharmacol 135: 1563–1571.

Krobert KA, Bach T, Syversveen T, Kvingedal AM, Levy FO (2001). The cloned human 5-HT7 receptor splice variants: a comparative characterization of their pharmacology, function and distribution. Naunyn Schmiedebergs Arch Pharmacol 363: 620–632.

Kroeze WK, Kristiansen K, Roth BL (2002). Molecular biology of serotonin receptors structure and function at the molecular level. Curr Top Med Chem 2: 507–528.

Kvachnina E, Liu G, Dityatev A, Renner U, Dumuis A, Richter DW *et al.* (2005). 5-HT7 receptor is coupled to G alpha subunits of heterotrimeric G12-protein to regulate gene transcription and neuronal morphology. J Neurosci 25: 7821–7830.

Lovell PJ, Bromidge SM, Dabbs S, Duckworth DM, Forbes IT, Jennings AJ *et al.* (2000). A novel, potent, and selective 5-HT(7) antagonist: (R)-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)pyrrolidine-1-sulfonyl) phen ol (SB-269970). J Med Chem 43: 342–345.

Lovenberg TW, Baron BM, de Lecea L, Miller JD, Prosser RA, Rea MA *et al.* (1993). A novel adenylyl cyclase-activating serotonin receptor (5-HT7) implicated in the regulation of mammalian circadian rhythms. Neuron 11: 449–458.

McAllister G, Charlesworth A, Snodin C, Beer MS, Noble AJ, Middlemiss DN *et al.* (1992). Molecular cloning of a serotonin receptor from human brain (5HT1E): a fifth 5HT1-like subtype. Proc Natl Acad Sci USA 89: 5517–5521.

Matsuoka M, Itoh H, Kaziro Y (1990). Characterization of the human gene for Gx alpha, a pertussis toxin-insensitive regulatory GTP-binding protein. J Biol Chem 265: 13215–13220.

Prasad MV, Dermott JM, Heasley LE, Johnson GL, Dhanasekaran N (1995). Activation of Jun kinase/stress-activated protein kinase by GTPase-deficient mutants of G alpha 12 and G alpha 13. J Biol Chem 270: 18655–18659.

Purohit A, Smith C, Herrick-Davis K, Teitler M (2005). Stable expression of constitutively activated mutant h5HT6 and h5HT7 serotonin receptors: inverse agonist activity of antipsychotic drugs. Psychopharmacology (Berl) 179: 461–469.

Raymond JR, Mukhin YV, Gelasco A, Turner J, Collinsworth G, Gettys TW *et al.* (2001). Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacol Ther 92: 179–212.

Roth BL, Craigo SC, Choudhary MS, Uluer A, Monsma FJ, Jr, Shen Y *et al.* (1994). Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors. J Pharmacol Exp Ther 268: 1403–1410.

Seamon KB, Padgett W, Daly JW (1981). Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc Natl Acad Sci USA 78: 3363–3367.

Shen Y, Monsma FJ, Jr, Metcalf MA, Jose PA, Hamblin MW, Sibley DR (1993). Molecular cloning and expression of a 5-hydroxytryptamine7 serotonin receptor subtype. J Biol Chem 268: 18200–18204.

Shukla AK, Violin JD, Whalen EJ, Gesty-Palmer D, Shenoy SK, Lefkowitz RJ (2008). Distinct conformational changes in beta-arrestin report biased agonism at seven-transmembrane receptors. Proc Natl Acad Sci USA 105: 9988–9993.

Smith C, Rahman T, Toohey N, Mazurkiewicz J, Herrick-Davis K, Teitler M (2006). Risperidone irreversibly binds to and inactivates the h5-HT7 serotonin receptor. Mol Pharmacol 70: 1264–1270.

Stengel D, Guenet L, Desmier M, Insel P, Hanoune J (1982). Forskolin requires more than the catalytic unit to activate adenylate cyclase. Mol Cell Endocrinol 28: 681–690.

Teitler M, Herrick-Davis K (1994). Multiple serotonin receptor subtypes: molecular cloning and functional expression. Crit Rev Neurobiol 8: 175–188.

Teitler M, Toohey N, Knight JA, Klein MT, Smith C (2010). Clozapine and other competitive antagonists reactivate risperidone-inactivated h5-HT7 receptors: radioligand binding and functional evidence for GPCR homodimer protomer interactions. Psychopharmacol (Berl) 212: 687–697.

Terron JA (1997). Role of 5-ht7 receptors in the long-lasting hypotensive response induced by 5-hydroxytryptamine in the rat. Br J Pharmacol 121: 563–571.

Thomas DR, Gittins SA, Collin LL, Middlemiss DN, Riley G, Hagan J *et al.* (1998). Functional characterisation of the human cloned 5-HT7 receptor (long form); antagonist profile of SB-258719. Br J Pharmacol 124: 1300–1306.

Toohey N, Klein MT, Knight J, Smith C, Teitler M (2009). Human 5-HT7 receptor-induced inactivation of forskolin-stimulated adenylate cyclase by risperidone, 9-OH-risperidone and other 'inactivating antagonists'. Mol Pharmacol 76: 552–559.

Tso PH, Yung LY, Wong YH (2000). Regulation of adenylyl cyclase, ERK1/2, and CREB by Gz following acute and chronic activation of the delta-opioid receptor. J Neurochem 74: 1685–1693.

Wong YH, Conklin BR, Bourne HR (1992). Gz-mediated hormonal inhibition of cyclic AMP accumulation. Science 255: 339–342.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Pertussis toxin (PTx) treatment has no affect on the activity of h5-HT_7 receptors stably expressed in HEK293 cells. Cells were cultured for 18 h in serum-free media with and without 100 ng·mL^{-1} PTx. Cells were then exposed to 10 µmol·L^{-1} 5-HT or 10 µmol·L–^{1} forskolin for 30 min and cAMP accumulation was determined. PTx treatment produced no significant affects on h5-HT_7 receptor activity (P = 0.412, two-way ANOVA). Data are the means ± SEM of three independent experiments performed in triplicate.

Figure S2 Inverse agonist efficacy does not correspond with the inhibition of forskolin-stimulated activity. (A) The inhibition of adenylate cyclase activity by 10 µmol·L^{-1} antagonist treatment of h5-HT_7-expressing cells was determined in the presence of forskolin [data from Figure 1: non-pertussis toxin (PTx)-treated cells] and in the absence of forskolin (i.e. inhibition of basal activity, inverse agonism). Except for cyproheptadine and bromocryptine, all antagonists significantly inhibited basal adenylate cyclase activity (P < 0.0001, one-way ANOVA with Dunnett's posttest). Two-way ANOVA found a significant interaction (P < 0.0001) between factor 1 (functional endpoint: inverse agonist efficacy, inhibition of forskolin) and factor 2 (drug: n = 14), indicating that maximal drug effects vary depending the functional endpoint measured. Data are normalized to 'No drug' control and are the means ± SEM of three independent experiments performed in triplicate. (B) Inhibition of forskolin-stimulated activity is plotted as a function of inverse agonist efficacy, demonstrating a lack of correlation (r^2 = 0.008) between these functional endpoints. Numbered points correspond to drug names listed.