Human 5-HT7 receptor-induced inactivation of forskolin-stimulated adenylate cyclase by risperidone, 9-OH-risperidone and other “inactivating antagonists”

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Abstract:

We have previously reported on the unusual h5-HT\textsubscript{7} receptor inactivating properties of risperidone, 9-OH-risperidone, bromocryptine, methiothepin, metergoline, and lisuride (Smith et al., 2006; Knight et al., 2009). Inactivation was defined as the inability of 10µM 5-HT to stimulate cAMP accumulation after brief exposure and thorough removal of the drugs from HEK293 cells expressing h5-HT\textsubscript{7} receptors. Herein we report that brief exposure of the h5-HT\textsubscript{7} receptor-expressing cells to inactivating drugs, followed by removal of the drugs, results in potent and efficacious irreversible inhibition of forskolin-stimulated adenylate cyclase activity. Pre-treatment, followed by removal of the inactivating drugs inhibited 10µM forskolin-stimulated adenylate cyclase activity with potencies similar to the drugs’ affinities for the h5-HT\textsubscript{7} receptor. The actions of the inactivating drugs were pertussis toxin-insensitive, indicating the lack of G\textsubscript{i} in their mechanism(s) of action. Methiothepin and bromocryptine maximally inhibited 10µM forskolin-stimulated adenylate cyclase while the other drugs produced partial inhibition, indicating the drugs are inducing slightly different inactive conformations of the h5-HT\textsubscript{7} receptor. Maximal effects of these inactivating drugs occurred within 15-30 min of exposure of the cells to the drugs. A G\textsubscript{s}-mediated inhibition of forskolin-stimulated activity has never been reported. The inactivating antagonists appear to induce a stable conformation of the h5-HT\textsubscript{7} receptor, which induces an altered state of G\textsubscript{s}, which, in turn, inhibits forskolin-mediated stimulation of adenylate cyclase. These and previous observations indicate the inactivating antagonists represent a unique class of drugs and may reveal GPCR regulatory mechanisms previously unknown. These drugs may produce innovative approaches to the development of therapeutically effective agents.
Introduction:

The 5-HT₇ receptor is one of 14 5-HT receptors expressed in mammalian tissues (Teitler and Herrick-Davis, 1994; Gerhardt and van Heerikhuizen, 1997; Hoyer and Martin, 1997; Hoyer et al., 2002; Kroeze et al., 2002; Raymond et al., 2001). It was discovered through homology cloning and is expressed in various areas of the human brain and in peripheral tissues, including important blood vessels in the cerebral vasculature (Bard et al., 1993; Lovenberg et al., 1993; Shen et al., 1993; Teitler and Herrick-Davis, 1994; Hedlund and Sutcliffe, 2004). 5-HT₇ receptor antagonists are being developed for possible use in various clinical conditions including migraine (Terron, 1997), sleep (Lovenberg et al., 1993), psychosis (Bard et al., 1993; Lovenberg et al., 1993; Shen et al., 1993), and depression (Bard et al., 1993; Lovenberg et al., 1993; Shen et al., 1993; Hedlund and Sutcliffe, 2004).

Risperidone is a highly prescribed atypical antipsychotic drug (Love and Nelson, 2000; Bhana and Spencer, 2000; Green, 2000; Schneider et al., 2006). It is one of a group of drugs believed to initiate their effects through interactions with the D₂ dopamine and 5-HT₂A serotonin receptors (Roth et al., 1994; Meltzer et al., 1989). These interactions have been shown to be classical competitive antagonist interactions (Smith et al., 2006; Roth et al., 1994). In previous publications, using h5-HT₇ receptor-expressing HEK293 cells, we reported the rapid, potent inactivation of h5-HT₇ receptor stimulation of cAMP production by six antagonists: risperidone, 9-OH-risperidone, methiothepin, bromocryptine, metergoline and lisuride (Smith et al., 2006; Knight et al., 2009). The mechanism appears to involve the pseudo-irreversible interaction of the drugs with the h5-HT₇ receptor, thus occluding the orthosteric binding site and preventing stimulation by 5-HT.

However, several observations indicated a simple pseudo-irreversible blockade might not fully explain the effect of the inactivating antagonists. Risperidone and 9-OH-risperidone irreversibly inhibited only 50% of the h5-HT₇ orthosteric binding sites, while the four other inactivators irreversibly inhibited
all the h5-HT7 receptor binding sites (Knight et al., 2009). Also, metergoline's potency as an inactivator was significantly lower than predicted from its affinity for the h5-HT7 receptor, while the other five inactivators' potencies matched their affinities for the h5-HT7 receptor.

The effects of forskolin on adenylate cyclase activity have been extensively studied (Stengel et al., 1982; Darfler et al., 1982; Insel and Ostrom, 2003; Alousi et al., 1991; Dessauer et al., 1997; Tang and Gilman, 1995). While the major effect of forskolin is to directly stimulate adenylate cyclase activity, this stimulation can be regulated by GPCRs through G-proteins (Tesmer et al., 2002; Taussig et al., 1993; Bender et al., 1984; Neer, 1986; Neer, 1978). The predominant regulation of forskolin-stimulated adenylate cyclase activity is mediated by activation of G\textsubscript{i}/\textsubscript{o}-coupled GPCR which partially inhibit forskolin-stimulated adenylate cyclase activity (Tesmer et al., 2002; Taussig et al., 1993; Bender et al., 1984; Neer, 1986; Neer, 1978). It has been reported that agonist-mediated modulation of forskolin-stimulated adenylate cyclase can be produced through G\textsubscript{s}-coupled receptors (Stengel et al., 1982). This effect is relatively minor and usually manifests as a potentiation of forskolin-stimulated activity. It should be noted that the GPCR-mediated regulation of forskolin-stimulated adenylate cyclase activity occurs through activity of agonists on GPCR. There appears to be no reports of acute effects of antagonists on forskolin-stimulated adenylate cyclase activity. While conducting the studies previously reported (Knight et al., 2009), forskolin-stimulated adenylate cyclase activity was routinely monitored as a control for the status of cellular adenylate cyclase activity. It was anticipated that forskolin-stimulated adenylate cyclase activity would not be affected by the inactivating drugs. As described below, the inactivating antagonists produced unique effects on h5-HT7 receptor activity, demonstrated by the persistent inhibition of forskolin-stimulated cAMP in cells exposed to this novel group of drugs. These effects provide significant information concerning the mechanism by which the inactivating antagonists produce their effects on h5-HT7 receptor-mediated cAMP production.
Materials and Methods:

**cAMP Assay:** Total cAMP accumulation was measured using the LANCE cAMP Detection kit (Perkin-Elmer). Cells were cultured for 18 hrs in serum-free media, with and without 100ng/ml pertussis toxin (see Results, figure 1). Cells were lifted using 1ml/dish diluted Versene (1:3 in PBS), followed by the addition of 11ml/dish HEPES buffer (20mM HEPES, 2.5mM MgSO4, 134mM NaCl, pH 7.5 at 23°C). Cells were centrifuged for 3 min at 330×g, supernatant was aspirated and the cells were resuspended in HEPES buffer. Cells were pre-treated with drug, incubated 30 min at 37°C (or 15, 30, 60, and 90 min for time-course experiments), washed 3×10 min in HEPES buffer. After the third wash, cells were resuspended in stimulation buffer (prepared according to the Perkin-Elmer LANCE cAMP instruction manual). Cells were counted with a hemacytometer and added to 96-well white opaque plates. The pre-treated cells were then exposed to 10µM or 35µM forskolin for 30 min at 23°C. Detection buffer was then added (prepared according to the LANCE instruction manual, Perkin-Elmer). Control experiments demonstrated that this procedure produced no effect on the cells' responsiveness to forskolin (see Results). Control experiments also demonstrated that performing the forskolin stimulation at 37°C has no effect on the properties of the inactivating antagonists (see Results). Time-resolved fluorescence resonance energy transfer was detected by the Victor3 1420 plate-reader (Perkin-Elmer).

**Risperidone and metergoline pre-treatment experiments:** Cells were lifted and centrifuged as above. Cells were incubated for 30 min with 10µM metergoline or 10µM risperidone at 37°C. The cells were then washed 3x10min with HEPES buffer. After the third wash cells were resuspended in HEPES buffer and treated with bromocryptine or methiothepin for 30 min at 37°C. Cells were then washed 3x10min with HEPES buffer. After the third wash, cells were resuspended in stimulation buffer (prepared according to the LANCE instruction manual, Perkin-Elmer). Cells were then counted using a
hematocytometer and added to 96-well opaque plates. The pre-treated cells were then exposed to 10µM forskolin for 30 min at room temperature. Plates were read on a Victor3 plate-reader (Perkin-Elmer).

Adenylate cyclase and Gαs immunoblots: Rabbit polyclonal A cyclase V/VI (H-130), Gαs (K-20), ERK2 and donkey anti-rabbit horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Suspended cells were treated with the specified drugs for 30 min at 37°C then washed (as described above) for 3x10 min with HEPES buffer. The cells were rinsed once with 1xPBS and spun 3 min at 330 x g. The cells were lysed for 30 min on ice with RIPA cell lysis buffer with protease inhibitors. The pellets were then further disrupted by shearing the DNA with a 26-gauge needle and another 30 min incubation on ice. The cell lysates were spun for 20 min at 14000 x g at 4 degrees. The supernatants containing the total cell lysate were flash frozen and stored at -80°C until needed. BCA’s were done on the cell treatments in order to determine the concentration of protein in each sample. 20µg of protein/treatment was loaded on to 10% Tris-HCL polyacrylamide gradient gels (Bio-Rad Laboratories, Hercules, CA) to be separated using electrophoresis and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were then blocked for one hour with 5% non-fat dry milk before overnight incubation with the primary antibody at 4°C. The membrane was washed and incubated with the secondary antibody and the bound antibodies were visualized using Pierce ECL western Blotting Substrate (Thermo Scientific, Rockford IL). The membranes were then washed, stripped, re-blotted with the ERK-2 antibody, and visualized as above.
Results:

Figures 1Aa and 1B displays the results of screening 19 drugs for forskolin-inactivating properties. These drugs were selected based on preliminary radioligand binding studies indicating they had high to moderate affinities for the h5-HT7 receptor. The h5-HT7 receptor expressing HEK293 cells were first exposed to 1µM drug for 30 minutes, followed by three washouts. The cells were then exposed to 10µM forskolin for 30 minutes. Inactivation was defined as the inability, or a reduced ability, of the cells to produce cAMP in response to forskolin stimulation after the thorough washout of drug. Six of the drugs tested displayed this property: risperidone, 9-OH-risperidone, methiothepin, bromocryptine, metergoline, and lisuride. These six drugs had been previously characterized as h5-HT7 receptor inactivators (Smith et al., 2006; Knight et al., 2009). The six drugs tested that had inactivating ability exhibited h5-HT7 receptor affinities that ranged from 0.4nM-143nM. Lisuride, (0.4nM), risperidone (2nM), methiothepin (3nM), 9-OH-risperidone (10nM), metergoline (16nM), and bromocryptine (143nM) displayed inactivating properties (figure 1A). Other drugs tested that did not display inactivating properties are listed with their h5-HT7 receptor affinities (nM) :amoxapine (69); amitriptyline (96); cyproheptadine (24); loxapine (258); mianserin (64); ritanserin (468); the selective 5-HT7 receptor antagonist SB269970 (2); tenilapine (153); TFM (1624); trifluperazine (497); the high affinity 5-HT2 receptor antagonist ICI169369 (393); clozapine (30); methysergide (32) and LSD (3). Of the six inactivators methiothepin and bromocryptine appeared to produce the most efficacious inhibition of 10µM forskolin-stimulation (figures 1). In order to determine if the h5-HT7 receptor might be stimulating a Gi, thereby inhibiting forskolin-stimulated adenylate cyclase activity, we pre-treated the h5-HT7-receptor expressing cells with pertussis toxin, which inactivates Gi (Kaslow et al., 1987). Although the h5-HT7 receptor is a well-characterized Gs-coupled receptor, it is possible that the "inactivating antagonists" are inducing a persistent state of the receptor that stimulates Gi, thereby inhibiting forskolin-
stimulated adenylate cyclase activity. As shown in figure 1A, pre-treatment of the h5-HT7-receptor expressing cells with pertussis toxin, produced no main effect on the inactivating antagonists' activity (p = 0.104, two-way ANOVA). The lack of effect of pertussis toxin indicates Gi is not involved in the inhibition of forskolin stimulated adenylate cyclase activity. Figure 1A displays the positive control for the pertussis toxin: 5-HT1E-mediated inhibition of forskolin-stimulated cAMP accumulation is blocked, indicating the pertussis toxin is active. Thus the lack of effect of pertussis toxin on the inhibition of forskolin-stimulated cAMP accumulation in the h5-HT7 receptor-expressing cells indicates no involvement of Gi. Figure 1B displays the lack of effect of 13 antagonists, i.e. “non-inactivating antagonists”, on forskolin-stimulated adenylate cyclase. Figure 1C displays the lack of effect of performing the assay at 37°, rather than 23°, on the inactivating antagonist drugs’ activity. Figure 2 displays the lack of effect of the inactivating antagonists on forskolin-stimulated adenylate cyclase activity in HEK-293 cells not expressing h5-HT7 receptors. Taken together the data in figures 1&2 indicate that six out of twenty drugs tested produced the inactivating effect on forskolin-stimulated adenylate cyclase activity, this effect is mediated through the h5-HT7 receptor rather than through a non-specific mechanism, and the effect is not due to some temperature-induced alteration in the assay conditions.

In order to obtain more information on this novel ability to irreversibly inhibit forskolin-stimulated adenylate cyclase activity, concentration-response curves for the inactivating effect were produced (figure 3; table 1). The h5-HT7 receptor expressing HEK293 cells were first exposed to increasing concentrations of drug for 30 minutes, followed by three washouts. The cells were then exposed to 10 or 35µM forskolin for 30 minutes. Risperidone, 9-OH-risperidone, bromocryptine, methiothepin, metergoline and lisuride displayed high potencies for producing the inactivation effect, with similar IC50 values for 10 or 35µM forskolin stimulation. The similar potencies of the inactivating drugs on 10 or 35µM forskolin-stimulated
activity (figure 3, table 1) indicate the mechanism-of-action is not a competitive one. An important observation from the data in figure 3 is that the inactivators appear to have different maximal levels of inhibition (table 2). Methiothepin and bromocryptine irreversibly inhibit forskolin-stimulated activity more effectively than risperidone, 9-OH-risperidone, metergoline or lisuride. These results indicate the possibility of either one mechanism with multiple efficacies among the inactivating drugs (full and partial inactivators), or multiple mechanisms of inhibition among the inactivating drugs. The major observation was that methiothepin and bromocryptine are fully efficacious in irreversibly inhibiting forskolin-stimulated adenylate cyclase activity, while the other four inactivating drugs display less efficacy.

In order to determine if the difference in efficacies could be due to kinetic differences between the drugs in producing the inactivating effect, time-course experiments were performed (figure 4). Cells were pre-treated with each of the inactivators for 15, 30, 60, and 90 minutes and then subjected to the usual washout and assay procedures (described above). While several interesting observations were made in these experiments (see Discussion) it is clear that the lower efficacy of risperidone, 9-OH-risperidone, metergoline and lisuride, relative to methiothepin and bromocryptine, cannot be due to slower kinetics. The lesser effect observed for several of the drugs at the 90 min time point is contrary to this possibility.

Another possible rationale for the difference in efficacies could be different mechanisms involving distinct sites on the h5-HT7 receptor mediating the effects of the inactivators. In order to obtain information on this possibility, h5-HT7 receptor expressing cells were exposed to 10µM metergoline or risperidone (partial inactivators) for 30 minutes, washed, and then exposed to 10µM methiothepin or bromocryptine (full inactivators). Metergoline and risperidone were chosen based on previous observations indicating they display somewhat different properties as inactivators (Knight et al., 2009). As shown in figure 5A, pre-treatment with metergoline resulted in a blunting of the effect of bromocryptine but had no effect on methiothepin's inactivating activity. Risperidone pre-treatment had no
effect on either methiothepin or bromocryptine (figure 5B). Taken together these results indicate that methiothepin, bromocryptine, risperidone, and metergoline produce inactivation by somewhat different mechanisms (see Discussion).

Figure 6 displays the results of Western blots for adenylate cyclase (isoforms V&VI) and $G_s$ (alpha subunit) subsequent to exposure of the h5-HT$_7$ receptor-expressing cells to no drug, 10µM clozapine, methiothepin or risperidone. None of the drugs produced any effect on the levels of adenylate cyclase or $G_s$, indicating that the loss of activity subsequent to inactivating drug treatment is not due to some dramatic effect on the cellular levels of these signal transduction components.

Discussion:

The results presented herein continue a series of unusual observations involving the h5-HT$_7$ receptor (Smith et al., 2006; Knight et al., 2009). Six drugs out of a total of 20 drugs that have been tested produce an irreversible inactivation of the h5-HT$_7$ receptor, as judged by inhibition of 10µM 5-HT stimulation of cAMP production. A pseudo-irreversible interaction between the "inactivating drugs" and the h5-HT$_7$ receptor appears to be the cause of this unusual effect (Smith et al., 2006). Five of the drugs appear to produce a complete inhibition of 5-HT stimulated receptor activity, with the possible exception of metergoline (Knight et al., 2009). The pseudo-irreversible block of the orthosteric binding site on the h5-HT$_7$ receptor appears to explain the inactivation of the receptor. However it was also noted that risperidone and 9-OH-risperidone, which potently and fully inactivate 5-HT stimulated h5-HT$_7$ receptor, only irreversibly block 50% of the binding sites (Knight et al., 2009). This observation indicates that receptor occupancy is not sufficient to fully predict the effect of the inactivators on the h5-HT$_7$ receptor.
activity. These results indicated that risperidone and 9-OH-risperidone, while producing a pseudo-
irreversible complex with the h5-HT7 receptor, might be, in addition, interacting with the h5-HT7 receptor
in a somewhat different manner than the other four inactivators.

In the present study another unusual observation is described. Forskolin-stimulated adenylate
cyclase activity is due to the direct interaction of forskolin with all the isoforms of adenylate cyclase,
except for the ram sperm form of the enzyme (Alousi et al., 1991). The interaction of activated Gs appears
to have a secondary influence on the ability of forskolin to stimulate adenylate cyclase (Tesmer et al.,
2002;Taussig et al., 1993;Bender et al., 1984;Neer, 1986;Neer, 1978). A more pronounced inhibitory
influence of Gt-coupled receptors on forskolin-stimulated adenylate cyclase is a well-documented cellular
mechanism (Mons and Cooper, 1995). However the involvement of Gi in the current study has been
eliminated, as pertussis toxin, an irreversible inhibitor of Gi, has no effect on the inactivating properties of
drugs presented (figure 1). Agonist stimulation of receptors, acting through GTP-binding proteins, have
been shown to slightly increase forskolin-stimulated adenylate cyclase activity (Darfler et al., 1982;Insel
and Ostrom, 2003;Alousi et al., 1991). Antagonist-induced inhibition of forskolin-stimulated adenylate
cyclase is a novel observation. The irreversible effects of the six inactivating drugs on forskolin-
stimulated adenylate cyclase activity, particularly that of methiothepin and bromocryptine, are
unprecedented. Furthermore these results indicate that the inactivating drugs, particularly methiothepin
and bromocryptine, must be doing more than simply irreversibly occluding the orthosteric binding site on
the h5-HT7 receptor. The effects on forskolin-stimulated adenylate cyclase suggest that the inactivators
are inducing a stable, persistent, inactive state of the h5-HT7 receptor that, in turn, is inducing an inactive
state of Gs. The inactive Gs must be inducing a state of adenylate cyclase with less than maximal
forskolin-stimulated potential. In other words, the effects on forskolin-stimulated activity observed reveal
several things about the receptor inactivating mechanism of these drugs. First, the inactivators irreversibly
induce a state of the receptor that inactivates G_s, eliminating the receptor-mediated cAMP stimulation as previously reported (Smith et al., 2006; Knight et al., 2009). Second, the G_s/adenylate cyclase interaction may be altered, inhibiting forskolin's ability to stimulate adenylate cyclase. The similar potencies of the inactivating drugs on 10 or 35µM forskolin-stimulated activity (figure 3, table 1) indicates the mechanism-of-action is not a competitive one. The maximal degree of irreversible inhibition of forskolin-stimulation varies amongst the different inactivators and is not predicted by maximal receptor occupancy (see Knight et al., 2009).

In order to determine the possibility that the lower maximal effect of metergoline was due to a slower onset of effect, time course experiments were performed (figure 4). Overall, the effect of prolonged exposure of cells to the inactivating drugs does not seem to have a major effect. However it was noted that metergoline did become less effective with prolonged exposure, especially in the 35µM forskolin experiments. This result is the opposite of what one would expect if the lower maximal effect of metergoline observed in figure 3 was due to a slower rate of onset for metergoline compared to the other inactivating drugs. The observed loss of effect of metergoline may indicate that this drug slowly dissociates, and after dissociation the receptor can become re-activated. This possibility is currently being investigated. There is a dramatic difference in the effect of 15 min lisuride exposure depending on whether 10 or 35µM forskolin is used as the stimulant (figure 4). This suggests that the effects of lisuride, at early time points may be reversed, by increasing forskolin concentrations. This possibility is also currently being investigated.

The results in figure 5A which shows metergoline pre-treatment blunts the effects of bromocryptine but does not blunt the effects of methiothepin are especially notable. These results are consistent with the complete blockade of the 5-HT_7 receptor by metergoline, preventing subsequent effects at the orthosteric site by bromocryptine. However, metergoline does not blunt the inactivating
effects of methiothepin. These results imply that bromocryptine and metergoline share the same mechanism, probably a pseudo-irreversible interaction with the h5-HT7 receptor. Methiothepin appears to have properties that allow it to overcome the presence of metergoline at the h5-HT7 receptor, i.e. methiothepin and bromocryptine induce different states of the h5-HT7 receptor that result in the complete inactivation of the h5-HT7 receptor.

The results in figure 5B, demonstrating that risperidone pre-treatment is ineffective in blunting the effects of either bromocryptine or methiothepin, are also notable. These results are consistent with previous results indicating that risperidone's mechanism of action is different than metergoline's (Knight et al., 2009). Risperidone and 9-OH-risperidone have been shown to completely inactivate the h5-HT7 receptor, while irreversibly blocking 50% of the receptors, while metergoline irreversibly blocks all the h5-HT7 receptors and produces a profound inactivation of the h5-HT7 receptor. The results in figure 4 reinforce the possibility of a different mechanism-of-action between risperidone and metergoline. Thus figure 4 indicates methiothepin, bromocryptine, risperidone, and metergoline differ in their interactions with the h5-HT7 receptor, as judged by forskolin-stimulated adenylate cyclase activity. The details of these differences are under investigation.

The results presented herein and in the two previous papers (Smith et al., 2006; Knight et al., 2009) are highly unusual and unprecedented. However there has been a published paper (Krobert et al., 2006) that reports similar effects with four drugs used in our studies. In this report the authors found that while mesulergine, SB269970, and clozapine produced little or no effect on the levels of 3H-5-CT-labeled h5-HT7 receptors after removal of the drugs, methiothepin reduced binding levels by 79%, with no change in affinity for the radioligand. These results correspond with the results we have reported previously: methiothepin inactivates the h5-HT7 receptor and inhibits 3H-5-HT labeling of the h5-HT7 receptor after
removal: mesulergine, SB269970 and clozapine do not produce these effects (Smith et al., 2006; Knight et al., 2009). We have not found any other studies that studied the 5-HT\textsubscript{7} receptor after removal of drugs.

The findings displayed in these studies, and in the previous papers {1,426} indicate that the h5-HT\textsubscript{7} receptor operates in a unique manner. While many antagonists behave in a classically competitive fashion, 6 out of the 20 antagonists tested appear to induce a stable state of the receptor that a) involves pseudo-irreversible binding; b) induces a stable inactivated state of G\textsubscript{s}, which in turn; c) induces a stable inactivated state of adenylate cyclase that includes complete or partial occlusion of the forskolin binding site. The inhibition of the forskolin-stimulated adenylate cyclase activity may not involve an occlusion of the binding site, but rather an altered, inactive conformation of adenylate cyclase that is resistant to forskolin stimulation. Studies are underway to investigate the predicted consequences of this model of h5-HT\textsubscript{7} function, i.e. a stable complex between the inactivating drug, h5-HT\textsubscript{7} receptor, G\textsubscript{s}, and adenylate cyclase.

In summary, the results presented add another novel observation concerning the effects of inactivating drugs on the h5-HT\textsubscript{7} receptor, through what appears to be a pseudo-irreversible complex (Smith et al., 2006; Knight et al., 2009). The irreversible inhibition of forskolin-stimulated activity provides strong evidence of the production of an inactivated state of G\textsubscript{s} by the inactivators, acting through the h5-HT\textsubscript{7} receptor. The different ability of the inactivators to inhibit forskolin-stimulated adenylate cyclase indicates the inactivators produce different states of the h5-HT\textsubscript{7} receptor. This situation is highly analogous to that of the classical case of full and partial receptor agonists adapted to this novel class of inactivating drugs. It is becoming increasingly clear that the inactivators are inducing a novel state of the h5-HT\textsubscript{7} receptor, revealing properties previously unobserved. As the effects of the inactivating drugs are examined in more detail, variations in the mechanism-of-action of these drugs are becoming clear. The
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inactivating drugs appear to possess properties that produce effects on GPCRs that are distinct from competitive antagonists and thus may lead to the discovery of novel GPCR regulatory mechanisms. These novel regulatory mechanisms may be targets for the development of novel therapeutic drugs.
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References:


Footnotes:

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Legends for Figures:

Figure 1: A: Effect of drug pre-treatment on 10µM forskolin-stimulated cAMP production by HEK 293 cells stably expressing h5-HT7 receptors, and the lack of effect of pertussis toxin on the drug effects. Cells were cultured for 18hr in serum-free media in the absence and presence of 100 ng/ml pertussis toxin. Cells were suspended in HEPES buffer and exposed to a 10µM concentration of drugs for 30 min. Cells were gently pelleted, and resuspended in HEPES buffer and incubated at 37°C for 10 min. This drug washout procedure was repeated three times. Cells were resuspended and assayed for response to 10µM forskolin using the LANCE cAMP Detection kit (Perkin-Elmer; see Materials and Methods). The results are the means ± SEM of three independent experiments performed in triplicate. Risperidone, 9-OH-risperidone, bromocryptine, methiothepin, lisuride, and metergoline were significantly different from no drug treatment (p<0.0001, one-way ANOVA). No significant main effect for pretussis toxin treatment was observed in h5-HT7 expressing cells (p = 0.104, two-way ANOVA). 5-HT1E receptor-expressing cells were tested for 5-HT-mediated inhibition of 10µM forskolin-stimulated cAMP production in the absence and presence of pertussis toxin (positive control).

B: Drugs displaying no effect on forskolin-stimulated adenylate cyclase activity after thorough washout. The results are the means ± SEM of three independent experiments performed in triplicate. There was no significant effect observed with these drugs (p=.12, one-way ANOVA).

C: Lack of effect of temperature on drug-induced inhibition of forskolin-stimulated adenylate cyclase activity. Cells were treated as in figure 1A except that forskolin stimulation was performed at 37°C rather than 23°C. There was no significant main effect on inhibition of forskolin-stimulated adenylate cyclase activity (p=0.45, two-way ANOVA).
Figure 2: Effect of drug pre-treatment on 10µM forskolin-stimulated cAMP production by parental HEK 293 cells. Cells were cultured for 18hr in serum-free media. Cells were suspended in HEPES buffer and exposed to a 10µM concentration of drugs for 30 min. Cells were gently pelleted, and resuspended in HEPES buffer and incubated at 37°C for 10 min. This drug washout procedure was repeated three times. Cells were resuspended and assayed for response to 10µM forskolin using the LANCE cAMP Detection kit (Perkin-Elmer; see Materials and Methods). The results are the means ± SEM of three independent experiments performed in triplicate.

Figure 3: Concentration-response curves for inactivation of 10µM and 35µM forskolin-stimulated cAMP production in HEK-293 cells expressing h5-HT7 receptors. Cells were suspended in HEPES buffer and exposed to buffer (control) or varying concentrations of the six drugs displaying inactivating properties (see figure 1). After the drug washout procedure (see Materials and Methods) the cells were exposed to 10µM or 35µM forskolin for 30 min. cAMP levels were determined using the LANCE cAMP Detection kit (Perkin-Elmer). The results are the means ± SEM of three independent experiments performed in triplicate. Dotted lines denote 10µM forskolin; solid lines denote 35µM forskolin.

Figure 4: Time course of maximal inhibition of forskolin-stimulated adenylate cyclase activity by h5-HT7 inactivators. Cells were exposed to 10µM inactivators for varying incubation times. After the drug washout procedure (see Materials and Methods) the cells were exposed to 10µM (A) or 35µM (B) forskolin and cAMP was detected as described above. Results are the means ±SEM of three independent experiments. While there was a significant effect of time on the drugs (p<0.001, two-way ANOVA), this effect does not account for the difference between the maximal effects of the different drugs (see Discussion).
Figure 5: Effect of metergoline or risperidone pre-treatment on inactivator inhibition of forskolin-stimulated adenylate cyclase. Cells were exposed to 10µM metergoline or risperidone for 30 minutes, thoroughly washed (see Materials and Methods), exposed to 10µM methiothepin or bromocryptine for 30 minutes, and thoroughly washed. After the drug washout procedure (see Materials and Methods) the cells were exposed to 10µM forskolin, and cAMP was detected as described above. Results are the means ±SEM of three independent experiments. Metergoline blunted the effects of bromocryptine (p<0.001) but had no effect on methiothepin’s activity. Risperidone had no significant effect on methiothepin or bromocryptine (see Discussion).

Figure 6: Levels of Gs and adenylate cyclase are not altered by inactivating drug exposure: Western blots using primary antibodies specific for Gs (α subunit) (A), and adenylate cyclase (sub-types V&VI) (B) were performed. There are no detectable differences between cells exposed to no drug (lane 1), 10µM clozapine (lane 2), 10 µM methiothepin (lane 3) or 10µM risperidone (lane 4). Cells were thoroughly washed before lysing and preparation. The blots were stripped after probing for either Gs or adenylate cyclase and re-probed for total ERK-2 as a loading control. Shown are representative blots that were performed twice.
Tables:

Table one: Potencies of the six h5-HT7 inactivating drugs as inhibitors of forskolin-stimulated adenylate cyclase activity. Following exposure of the h5-HT7-receptor expressing HEK293 cells to varying concentrations of drugs for 30 min, the drugs were removed by repeated washing. Activity was determined by exposing the cells to 10 or 35µM forskolin for 30 min. cAMP was detected using the LANCE cAMP detection kit (see Materials and Methods). Results are the means ± SEM of three independent experiments performed in triplicate. Also included are the Ki values determined from homogenate binding studies (Knight et al., 2009). There was no effect of increasing forskolin concentration on drug potencies (p = .492, two-way ANOVA).

<table>
<thead>
<tr>
<th>Drug</th>
<th>K_i (nM)</th>
<th>IC50 (10µM forskolin)</th>
<th>IC50 (35µM forskolin)</th>
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<tbody>
<tr>
<td>risperidone</td>
<td>1.8±0.3</td>
<td>4.4±2.1nM</td>
<td>3±0.9nM</td>
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<tr>
<td>9-OH risperidone</td>
<td>10±1.7</td>
<td>4.5±0.9nM</td>
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<td>bromocryptine</td>
<td>143±56</td>
<td>172±13nM</td>
<td>187±3.4nM</td>
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<tr>
<td>lisuride</td>
<td>0.4±0.2</td>
<td>2.9±0.7nM</td>
<td>1.8±0.1nM</td>
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<tr>
<td>metergoline</td>
<td>16±2</td>
<td>394±64nM</td>
<td>306±17nM</td>
</tr>
<tr>
<td>methiothepin</td>
<td>3.0±0.5</td>
<td>3.0±0.4nM</td>
<td>2.5±0.2nM</td>
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</table>

*a membrane homogenate binding; (Knight et al., 2009)
Table two: Maximal effects of the six inactivating drugs on 10 and 35µM forskolin-stimulated activity. h5-HT7-receptor expressing cells were exposed to 10µM drugs for 30 min, thoroughly washed, then exposed to 10 or 35µM forskolin for 30 min (see figure three). The values listed are the percentage of forskolin-stimulated activity observed in cells not exposed to inactivators. cAMP was detected using the LANCE kit (see Materials and Methods). Results are the mean±SEM of three independent experiments.

<table>
<thead>
<tr>
<th>Inactivator (10uM)</th>
<th>10uM forskolin (% control)</th>
<th>35uM forskolin (% control)</th>
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<tbody>
<tr>
<td>risperidone</td>
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<td>25±2</td>
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<td>9-OH-risperidone</td>
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<tr>
<td>metergoline</td>
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<tr>
<td>lisuride</td>
<td>25±4</td>
<td>32±8</td>
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**Figure 1A**

![Bar chart showing cAMP levels in h5-HT7 receptors with and without PTx treatment.](image)

**Figure 1B**

![Bar chart showing cAMP levels in h5-HT1E receptors.](image)

**Figure 1C**

![Bar chart showing cAMP levels at 37°C.](image)
Figure 2: Graph showing the effect of various drugs on cAMP levels in parental HEK-293 cells. The y-axis represents cAMP (% of 10 μM forskolin), and the x-axis lists different drugs: no drug, risperidone, 9-OH-risperidone, bromocriptine, methiothepin, lisuride, metergoline.
Figure 4A

Figure 4B
### A.

<table>
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### B.

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