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[^{3}H]5-Hydroxytryptamine labels the agonist high affinity state of the cloned rat 5-HT$_4$ receptor [1]

Nika Adham [a,*], Christophe Gerald [a], Lee Schechter [b], Pierre Vaysse [a], Richard Weinshank [a], Theresa Branchek [a]

[a] *Synaptic Pharmaceutical Corporation, 215 College Road, Paramus, NJ 07652, USA*
[b] *Wyeth-Ayerst Research, CNS Disorders, CN8000, Princeton, NJ 08543, USA*

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Abstract

We have used the cloned rat 5-HT$_4$ receptor, and determined that the single protein product produced is able to bind both [^{3}H]5-HT and [^{3}H]GR113808 ([1-[(2-methyl sulphonyl) amino] ethyl-4-piperidinyl] methyl-1-methyl-1*H*-indole-3-carboxylate) with high affinity. The affinities of agonists for the [^{3}H]5-HT (agonist)-labelled receptor were significantly higher than for the [^{3}H]GR113808 (antagonist)-labelled receptor. Furthermore, [^{3}H]5-HT binding was reduced by addition of guanyl nucleotides. These results strongly support the hypothesis that the 5-HT$_4$ receptor displays two interconvertible affinity states (high and low) for agonists, characteristic of many G protein coupled receptors. [^{3}H]5-HT, at the concentration employed, therefore labels the agonist high affinity state of the 5-HT$_4$ receptor in systems in which high densities of this receptor are found.

Keywords: 5-HT$_4$ receptor; Splice variant; Affinity state; Radioligand binding

1. Introduction

Until the recent development of [^{3}H]GR113808 ([1-[(2-methyl sulphonyl) amino] ethyl-4-piperidinyl] methyl-1-methyl-1*H*-indole-3-carboxylate) as a high affinity receptor antagonist radioligand, the classification of the 5-HT$_4$ receptor had been based solely on functional responses mediated by this subtype (Ford and Clarke, 1993). The availability of a radioligand for the 5-HT$_4$ receptor has greatly facilitated more detailed studies of this receptor including its distribution in brain and periphery of several species (Grossman et al., 1993; Waeber et al., 1993).

In rat brain homogenates, some investigators have observed that agonist competition curves for [^{3}H]GR113808 are shallow, and that GTP and related analogs cause a steepening and rightward shift of this competition curve, indicating the conversion of some receptor agonist high affinity states to the receptor agonist low affinity conformation (Grossman et al., 1993). These observations provide indirect evidence for the presence of agonist low and high affinity states of the 5-HT$_4$ receptor. In the present study, we show that [^{3}H]5-HT could be used to label the high affinity state of the 5-HT$_4$ receptor provided that the receptor is expressed at a fairly high density. The isolation of two putative splice variants of the rat 5-HT$_4$ receptor gene (5-HT$_{4S}$ and 5-HT$_{4L}$) has beeen reported by Gerald et al. (1994, 1995). Competition studies using [^{3}H]GR113808 as the radioligand have shown that these two receptors have almost identical pharmacological profiles (Gerald et al., 1995). However, we chose to focus our study on the 5-HT$_{4L}$ splice variant because functional studies in Cos-7 cells indicated that it had a better coupling efficiency for stimulating adenylyl cyclase and also agonist binding to this receptor was more sensitive to modulation by guanine nucleotides in comparison with 5-HT$_{4S}$ receptor (Adham et al., 1995).

2. Materials and methods

2.1. Transfections

The full coding region of 5-HT$_{4L}$ (Gerald et al., 1995) was subcloned in the mammalian expression vector

* Corresponding author. Tel.: (201) 261-1331; fax: (201) 261-0623.

[1] A preliminary report of some of the work described in this paper has been previously presented (Adham et al., 1994).

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pEXJ.BS (Miller and Germain, 1986; Okayama and Berg, 1983). For transient expression, Cos-7 cells were transfected by the diethyl amino ethyl-dextran (DEAE-dextran) method, using 1 μg of DNA/10^6 cells (Cullen, 1987).

2.2. Radioligand binding studies

Membranes used in the radioligand binding assays were prepared from transiently transfected cells, using a procedure described previously (Branchek et al., 1990). Protein concentrations were determined by the method of Bradford (1976). Radioligand binding studies were performed at 37°C in a total volume of 250 μl of buffer (50 mM Tris[hydroxymethyl]aminomethane (Tris), 10 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 10 μM pargyline, 0.1% ascorbate, pH 7.4 at 37°C) in 96 well microtiter plates. Saturation studies were conducted using 8–10 concentrations of either [^{3}H]5-HT (5–100 nM) or [^{3}H]GR113808 (0.005–2.5 nM). Displacement studies were performed using either 5–10 nM [^{3}H]5-HT or 0.2–0.4 nM [^{3}H]GR113808 and 10–12 concentrations of the competitor. Incubation times were 30 min for both saturation and displacement studies. Nonspecific binding was defined by 10 μM (for [^{3}H]5-HT binding) or 50 μM (for [^{3}H]GR113808 binding) unlabelled 5-HT. Binding was initiated by the addition of 50 μl membrane homogenates (10–20 μg protein). The reaction was terminated by rapid filtration through presoaked (0.5% polyethyleneimine) filters using 48R Cell Brandel Harvester (Gaithersburg, MD, USA). Subsequently, filters were washed for 5 s with ice-cold buffer (50 mM Tris HCl, pH 7.4 at 4°C), dried and placed into vials containing 2.5 ml of Ready-Safe (Beckman, Fullerton, CA), and radioactivity was measured using a Beckman LS 6500C liquid scintillation counter. The efficiency of counting of [^{3}H]GR113808 averaged between 45–50%.

2.3. Data analysis

Binding data were analyzed by computer-assisted non-linear regression analysis (Accufit and Accucomp, Lundon Software, Chagrin Falls, OH). IC_{50} values were converted to K_i values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

2.4. Materials

Drugs were obtained from the following sources: [^{3}H]5-HT (20–30 Ci/mmol), New England Nuclear (Boston, MA, USA); [^{3}H]GR113808 (80–85 Ci/mmol), Amersham International (Arlington Heights, IL, USA); 5-HT and guanosine-5′-*O*-(3-thiotriphosphate) (GTPγ-S), Sigma Chemicals (St. Louis, MO, USA); 5-methoxytryptamine, α-methyl 5-HT, 5-carboxamidotryptamine (5-CT), renzapride (BRL-24924), zacopride, tropisetron (ICS205930), 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) and ketanserin, Research Biochemicals (Natick, MA, USA).

3. Results

The 5-HT_{4L} receptor expressed in Cos-7 cells displays reliable sensitivity to modulation by GTP analogues and thus provides a suitable model to study the agonist high affinity state of the 5-HT_4 receptor. In Cos-7 cells transfected with the 5-HT_{4S} receptor gene, GTPγ-S at a final concentration of 100 μM produced no significant effect on the 5-HT displacement of specific [^{3}H]GR113808 binding (K_i control, 116 ± 18 nM; K_i GTPγ-S, 158 ± 32 nM ($n = 2$)). On the other hand, in the case of the 5-HT_{4L} receptor, GTPγ-S produced a small (2-fold) but significant reduction in the affinity of 5-HT (K_i control, 64 ± 4 nM; K_i GTPγ-S, 119 ± 13 nM ($n = 2$, $P < 0.05$)) (Gerald et al., 1995).

Both [^{3}H]5-HT and [^{3}H]GR113808 exhibited high affinity and saturable binding to transiently transfected

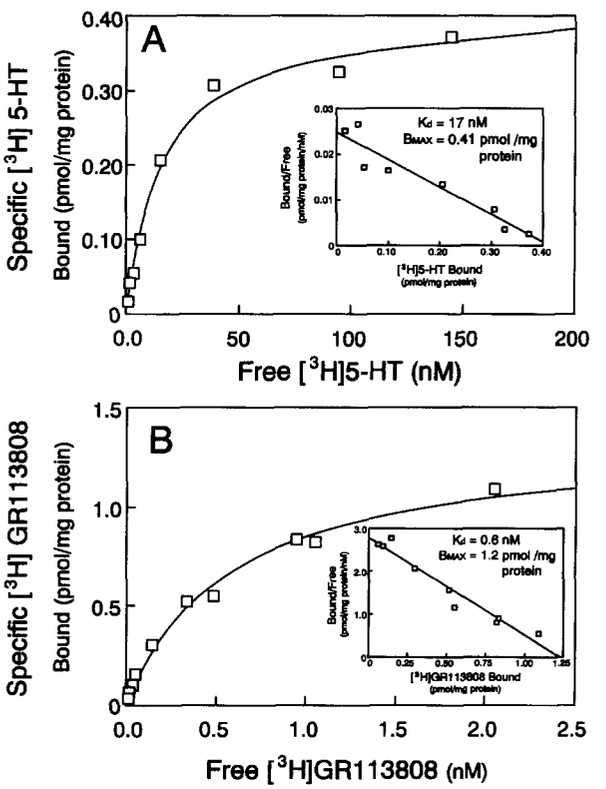

Fig. 1. Saturation analysis of (A) [^{3}H]5-HT and (B) [^{3}H]GR113808 binding to the cloned rat 5-HT_{4L} receptor. Membranes harvested from transiently transfected Cos-7 cells were incubated with 8–10 concentrations of either [^{3}H]5-HT (5–100 nM) or [^{3}H]GR113808 (0.005–2.5 nM) for 30 min at 37°C. Non-specific binding was defined by 10 μM (for [^{3}H]5-HT binding) or 50 μM (for [^{3}H]GR113808 binding) unlabeled 5-HT. Results are from one representative experiment. K_d and B_{max} values were determined by computer-assisted non-linear regression analysis (Accufit; Lundon Software) and for each radioligand, these values are illustrated in the form of a Scatchard plot (inset).

Cos-7 cells expressing the rat 5-HT_{4L} receptor (Fig. 1) but neither radioligand showed any specific binding to mock-transfected cells (data not shown, Adham et al., 1994). The specific [^{3}H]5-HT and [^{3}H]GR113808 binding represented approximately 50% and 90% of the total binding, respectively, at a ligand concentration equal to their equilibrium dissociation constants. The measured dissociation constants for [^{3}H]5-HT and [^{3}H]GR113808 were 20 ± 7 nM (n = 3) and 0.75 ± 0.12 nM (n = 5), respectively. Evaluation of the Scatchard transformation yielded a single binding site irrespective of the radioligand used (Fig. 1). The mean density of [^{3}H]5-HT binding sites (B_{max}) was 0.32 ± 0.10 pmol/mg protein (n = 3) and averaged 15–30% of the total sites labelled by [^{3}H]GR113808 (B_{max} = 1.7 ± 0.5 pmol/mg protein (n = 5)).

Pharmacological characterization of [^{3}H]5-HT and [^{3}H]GR113808 binding was obtained by analysis of competition binding experiments. Specific binding for both radioligands was inhibited only by compounds that have been shown to be active at the pharmacologically-defined 5-HT_4 receptor (Table 1). Other subtype selective compounds such as 8-OH-DPAT (5-HT_{1A}), sumatriptan (5-HT_{1D}), ketanserin (5-HT_2) and D-lysergic acid diethylamide (D-LSD) (5-HT_1, 5-HT_2, 5-HT_5, 5-HT_6 and 5-HT_7) at a concentration of up to 1 μM had no effect on the specific binding of either [^{3}H]5-HT or [^{3}H]GR113808 (data not shown).

All receptor agonists displayed 4–20-fold higher affinity for [^{3}H]5-HT than for [^{3}H]GR113808 binding based on one-site fit of displacement curve. The troponyl derivative, ICS205930, which has been shown to be a competitive antagonist at the pharmacologically defined 5-HT_4 receptor also showed slightly higher affinity (approximately 3-fold) for the agonist-labelled site (Table 1). Agonist displacement curves for [^{3}H]5-HT binding had Hill slopes close to unity (Fig. 2A) whereas competition of [^{3}H]GR113808 binding displayed shallow competition curves with Hill numbers less than unity (5-HT curve competition curve, n_H = 0.65–0.74) with approximately 20–30% of the sites in the high affinity state (Fig. 2B). Addition of the nonhydrolyzable analogue of GTP, GTPγ-S (100 μM), resulted in the steepening of the slope (n_H = 0.80–0.90) and a rightward shift of the competition curve, reducing the K_i of 5-HT by approximately 2-fold (see above and also Gerald et al., 1995). At a concentration of [^{3}H]5-HT equivalent to its K_d value, agonist binding to the receptor was inhibited < 50% (49 ± 15%, n = 3) by GTPγ-S with a very low affinity (K_i > 10 μM) (Fig. 3). Furthermore, the degree of GTPγ-S-induced interconversion and the ratio of high to low affinity states in different cell culture harvests were variable. For example, GTPγ-S-induced effects were only observed in 2 experiments out of a total of 4 that were performed.

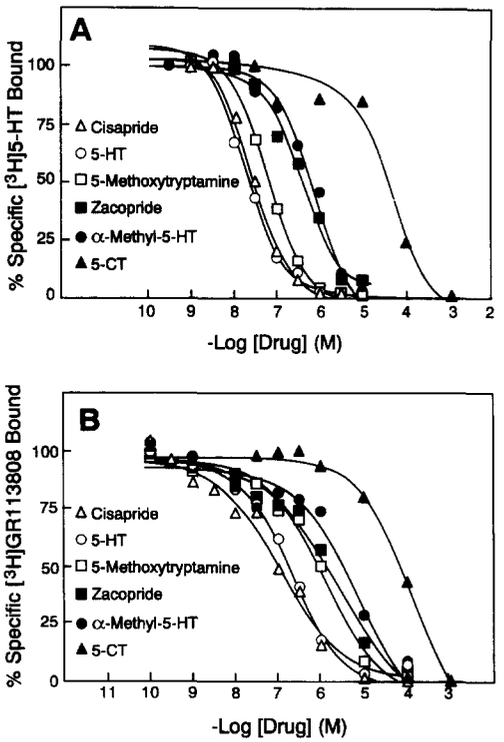

Fig. 2. Inhibition of specific (A) [^{3}H]5-HT and (B) [^{3}H]GR113808 binding to the cloned rat 5-HT_{4L} receptor. Membranes from transiently transfected Cos-7 cells were incubated with 10 nM [^{3}H]5-HT or 0.4 nM [^{3}H]GR113808 in the presence or absence of various drugs for 30 min at 37°C. Results are presented as a percentage of specific binding in the absence of a competing agent. Data are from a single experiment that is representative of 3 such experiments.

Table 1
The affinities of various compounds that compete for 8–10 nM [^{3}H]5-HT and 0.2–0.4 nM [^{3}H]GR113808 binding at the cloned 5-HT_{4L} receptor. Affinity values are given as K_is in nM and were determined from IC_{50} values obtained by computer-assisted non-linear curve analysis (Accucomp; Lundon Software), using the Cheng-Prussoff equation (Cheng and Prusoff, 1973). K_i values are expressed as means ± S.E.M. from at least 3 determinations

Compound	K_i (nM) [^{3}H]5-HT	K_i (nM) [a] [$_3$H]GR113808
Agonists		
5-HT	6.3 ± 0.5	145 ± 30
5-Methoxytryptamine	112 ± 16	401 ± 69
α-Methyl-5-HT	263 ± 34	1450 ± 406
5-CT	> 1000	> 1000
Cisapride	25 ± 9	122 ± 38
BRL-24924	27 ± 6	243 ± 28
Zacopride	136 ± 6	808 ± 79
Antagonist		
ICS205930	159 ± 5	544 ± 15

[a] From Gerald et al. (1995).

4. Discussion

We have recently isolated two putative splice variants (5-HT_{4S} and 5-HT_{4L}) of the gene encoding the pharmaco-

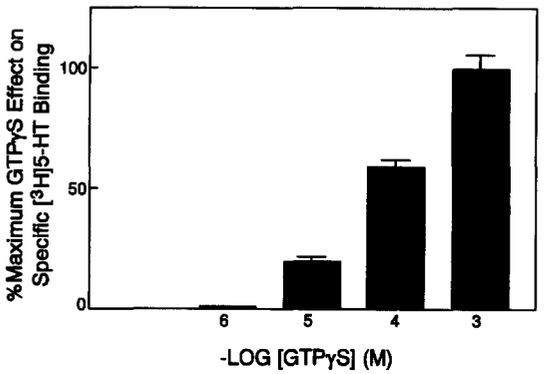

Fig. 3. Effect of GTPγ-S on [^{3}H]5-HT binding to the cloned rat 5-HT$_{4L}$ receptor. Membranes from transiently transfected Cos-7 cells were incubated with 10 nM [^{3}H]5-HT in the absence or presence of indicated concentrations of GTPγ-S for 30 min at 37°C. Results are presented as percentages of maximum inhibition of [^{3}H]5-HT binding produced by GTPγ-S. Data are averages of triplicate determinations, with standard errors < 10%.

logically defined 5-HT$_4$ receptor (Gerald et al., 1994, 1995). The amino acid sequences of these receptor isoforms are identical in the transmembrane region and differ only in the length and sequence of their carboxy termini. When these receptors were expressed transiently in Cos-7 cells at a high density the pharmacological profile obtained using [^{3}H]GR113808 was very similar to those reported for the 5-HT$_4$ receptor in brain tissue of several species (Grossman et al., 1993; Waeber et al., 1993). Furthermore, affinities of several compounds for displacing specific [^{3}H]GR113808 binding were almost identical for 5-HT$_{4S}$ and 5-HT$_{4L}$ receptors (Gerald et al., 1994). However, since effects of GTP analogues on agonist binding to the receptor were more reproducible for 5-HT$_{4L}$ as compared to 5-HT$_{4S}$ we chose to evaluate affinity states of the 5-HT$_{4L}$ receptor.

Radioligand binding studies in rat brain tissues, using the potent 5-HT$_4$ selective antagonist, [^{3}H]GR113808, have been consistent with the hypothesis that the 5-HT$_4$ receptor exists in a high (G protein coupled) and a low affinity state for agonists (Grossman et al., 1993). In native tissues, the fraction of 5-HT$_4$ receptors coupled to G proteins (agonist high affinity sites of the receptor) is most likely to be small due to the low density of these receptors (20–80 fmol/mg membrane protein as measured by [^{3}H] GR113808). This consideration, in conjunction with the lack of a selective high affinity receptor agonist radioligand with a high specific activity, has made it impossible to demonstrate direct labelling of the high affinity state of the 5-HT$_4$ receptor in these preparations. In order to study the agonist and antagonist binding properties of the 5-HT$_4$ receptor, we transfected the cloned rat 5-HT$_{4L}$ receptor in Cos-7 cells and were able to obtain a significantly higher density (approximately 2 pmol/mg membrane protein as measured by [^{3}H]GR113808 binding) of this receptor compared to native systems. Expression of this single receptor cDNA also led to the appearance of saturable, high affinity [^{3}H]5-HT binding sites that averaged 15–30% of those sites labelled with the antagonist, [^{3}H]GR113808. It is also important to note that receptor agonists inhibited the binding of [^{3}H]5-HT to the receptor with higher affinity (approximately 4–20-fold) than that observed when [^{3}H]GR113808 was used as a radioligand. These results are based on one-site analysis at the [^{3}H]GR113808 displacement curves which gives an overall K_i value that is a composite of the affinity of both the high and the low affinity sites of the receptor for agonists. This difference in affinities would be expected to be magnified if the K_i obtained from receptor agonist-labelled site (single high affinity site) is compared with the K_i of agonists obtained in the presence of GTP analogues (agonist low affinity site). However, the pure agonist low affinity state of the receptor could not be achieved due to the partial sensitivity of the agonist binding to GTP analogues. The incomplete conversion of agonist high to low affinity sites seen here has been described in many other systems and may be attributed to a variety of factors including receptor oxidation (Emerit et al., 1991) and/or coupling of the receptor to G proteins that have poor sensitivity and affinity for GTP (Szele and Prichett, 1993). In systems where a receptor is very efficiently coupled to G proteins, GTPγS inhibits agonist binding to the receptor with a K_i of < 100 nM (Szele and Pritchett, 1993).

It is noteworthy that the competitive antagonist, ICS205930, also showed slightly higher (approximately 3-fold) affinity for the receptor agonist- than the antagonist-labelled site. The finding with ICS205930 is more consistent with the properties of a weak partial agonist for which agonistic behaviour depends on the degree of receptor reserve in a given system. ICS205930 has been shown by Corsi et al. (1991) to have some weak intrinsic activity at the 5-HT$_4$ receptor in the electrically stimulated isolated human urinary bladder.

The data presented herein demonstrate that the single protein product produced by transient transfection of the rat 5-HT$_{4L}$ cDNA in an heterologous expression system is able to bind both [^{3}H]5-HT and [^{3}H]GR113808 with high affinity. These results are reminiscent of the [^{3}H]agonist vs [^{3}H]antagonist binding properties of the cloned 5-HT$_{2A}$ receptor (Branchek et al., 1990; Teitler et al., 1990) and for the first time indicate that [^{3}H]5-HT can label the agonist high affinity state of the 5-HT$_4$ receptor under conditions of high receptor expression. Such high affinity states could be present in preparations of native 5-HT$_4$ receptors although they would be difficult to assess using non-selective agonists such as 5-HT. Direct visualization of agonist high affinity 5-HT$_4$ sites in native tissues still awaits development of a selective (and probably iodinated) 5-HT$_4$ receptor agonist.

It should be emphasized that the observations made here are in systems that are unnatural for the receptor (high receptor densities, the receptor may not be coupling to its

natural G protein, the receptor/G protein stoichiometry may be very different from that found in native tissues, etc...) and may not precisely reflect as to how 5-HT_4 receptors are naturally coupled to G proteins in native tissues. However, these systems are the first to allow the direct measurement of the agonist high affinity state of the 5-HT_4 receptor and may thus provide a tool for studying the relationship between the agonist high affinity state and the functional responses produced by these receptors. Further studies are required to understand factors leading to the variability of the apparent GTP sensitivity and the apparent differences in the sensitivity of the two putative 5-HT_4 splice variants. Production of well-coupled stable cell lines for use in such a study is in progress.

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