ELSEVIER

European Journal of Pharmacology 361 (1998) 299–309

ejp

The human 5-ht$_{5A}$ receptor couples to G_i/G_o proteins and inhibits adenylate cyclase in HEK 293 cells

Bart J.B. Francken [a,*], Mirek Jurzak [a], Jurgen F.M. Vanhauwe [a], Walter H.M.L. Luyten [b], Josée E. Leysen [a]

[a] *Department of Biochemical Pharmacology, Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse, Belgium*
[b] *Department of Experimental Molecular Biology, Janssen Research Foundation, B-2340 Beerse, Belgium*

Received 13 August 1998; revised 7 October 1998; accepted 13 October 1998

Abstract

The G protein coupling of human 5-hydroxytryptamine$_{5A}$ (h5-ht$_{5A}$) receptors was investigated in stably transfected human embryonic kidney (HEK) 293 cells, using radioligand and guanosine-5′[γ-^{35}S]thiotriphosphate binding to membranes and cyclic adenosine monophosphate measurements in cells. 5-Carboxamido[^{3}H]tryptamine bound to high- and low-affinity sites on h5-ht$_{5A}$-HEK 293 cell membranes. Guanylyl-imidodiphosphate addition and pertussis toxin pre-treatment abolished high-affinity binding, indicating coupling to G proteins of the G_i/G_o family. [*N*-methyl-^{3}H]Lysergic acid diethylamide bound to a single site; guanylyl-imidodiphosphate and pertussis toxin did not alter lysergic acid diethylamide affinity. 5-Hydroxytryptamine stimulated guanosine-5′[γ-^{35}S]thiotriphosphate binding to 130% over basal and this effect was completely abolished by pertussis toxin. Various 5-hydroxytryptamine receptor ligands were tested for inhibition of 5-carboxamido[^{3}H]tryptamine binding and in guanosine-5′[γ-^{35}S]thiotriphosphate binding assays. 5-Hydroxytryptamine consistently inhibited forskolin-induced cyclic adenosine monophosphate formation by 25% in h5-ht$_{5A}$-HEK 293 cells; no effect was detected on basal cyclic adenosine monophosphate levels, on intracellular Ca^{2+} concentration or arachidonic acid release. Our studies demonstrate functional coupling of the h5-ht$_{5A}$ receptor to pertussis toxin-sensitive G proteins and to inhibition of adenylate cyclase activity.

Keywords: 5-ht$_{5A}$ receptor; Human; G protein; Pertussis toxin; Guanosine-5′[γ-^{35}S]thiotriphosphate binding; Cyclic adenosine monophosphate

1. Introduction

The neurotransmitter 5-hydroxytryptamine (5-HT, serotonin) is involved in the control of diverse physiological processes including sleep, sexual behaviour, food intake, locomotion and mood. Depression, schizophrenia and migraine are among the pathological conditions associated with a dysfunction of 5-HT transmission.

At least thirteen different 5-HT receptors have been identified to date. One, the 5-HT$_3$ receptor, belongs to the superfamily of ligand-gated ion channels; the others are seven-transmembrane-domain receptors that couple to heterotrimeric guanine nucleotide-binding proteins (G proteins). The G protein-coupled 5-HT receptors are classified into six subfamilies (5-HT$_1$, 5-HT$_2$, 5-HT$_4$, 5-ht$_5$, 5-ht$_6$ and 5-HT$_7$) (for review, Saudou and Hen, 1994; Hoyer and Martin, 1997).

The two members of the 5-ht$_5$ receptor subfamily, 5-ht$_{5A}$ and 5-ht$_{5B}$, were first identified in mice (Plassat et al., 1992; Matthes et al., 1993) and subsequently in rats (Erlander et al., 1993; Wisden et al., 1993). A cDNA encoding the 5-ht$_{5A}$ receptor has been cloned from human tissue, while the 5-ht$_{5B}$ receptor does not seem to be functionally expressed in humans (Rees et al., 1994; Grailhe et al., 1995). Pharmacologically, 5-ht$_5$ receptors resemble the 5-HT$_1$ subfamily of receptors, displaying high affinities for the agonist 5-CT and ergot derivatives, such as LSD and ergotamine. However, for various reasons 5-ht$_5$ receptors may represent a distinct subfamily. In contrast to 5-HT$_1$ receptor genes, 5-ht$_5$ genes contain an intron in the region encoding the third intracellular loop (Plassat et al., 1992). Furthermore, 5-ht$_5$ receptors exhibit

* Corresponding author. Tel.: +32-14-60-27-13; Fax: +32-14-60-53-80; E-mail: bfrancke@janbe.jnj.com

0014-2999/98/$ - see front matter
PII: S0014-2999(98)00744-4

a rather low affinity for 5-HT and the percentage of amino acid sequence homology in the transmembrane domains to other 5-HT receptors is less than 50% (Humphrey, 1997).

The physiological function of 5-ht$_5$ receptors is still unclear. At present, no selective ligands are available. Possible functions have been postulated based on localisation studies, using Northern blot analysis, reverse transcriptase-polymerase chain reaction, in situ hybridisation and immunohistochemistry. For example, the limbic distribution (dendate gyrus and CA1/CA3 regions of the hippocampus, amygdala, entorhinal cortex) of 5-ht$_{5A}$ mRNA in the mouse, rat and human brain suggests a possible role in learning, memory and emotional behaviour (Plassat et al., 1992; Erlander et al., 1993; Pasqualetti et al., 1998), while the presence of 5-ht$_{5A}$ mRNA in the cerebellum may implicate a role in motor co-ordination and control (Plassat et al., 1992; Carson et al., 1996; Pasqualetti et al., 1998). 5-ht$_{5A}$ mRNA was also detected in the habenula (Plassat et al., 1992; Matthes et al., 1993), suggesting a possible role for 5-ht$_{5A}$ receptors in the acquisition of adaptive behaviour under stressful situations (Branchek and Zgombick, 1997). Recently, knockout mice lacking the 5-ht$_{5A}$ receptor were generated. These mutant mice were shown to have enhanced exploratory activity in a novel environment, suggesting that 5-ht$_{5A}$ receptors might modulate exploratory behaviour (Grailhe et al., 1997).

The mouse, rat and human 5-ht$_5$ receptors have already been expressed in various cell systems, but most of these reported studies failed to demonstrate effects on signal transduction systems, such as adenylate cyclase or phospholipase C (Plassat et al., 1992; Matthes et al., 1993; Erlander et al., 1993; Wisden et al., 1993). Although no second messenger coupling could be detected for the mouse 5-ht$_{5A}$ receptor, a GTP-sensitive binding site was described when the receptor was expressed at low density in NIH 3T3 cells (Plassat et al., 1992). Furthermore, high-affinity agonist radioligand ([^{3}H]5-CT) binding to the rat 5-ht$_{5B}$ receptor expressed in COS-1 cells could be shifted by the addition of Gpp(NH)p (Wisden et al., 1993). Recently, however, the rat 5-ht$_{5A}$ receptor expressed in C6 glioma cells was reported to be negatively coupled to adenylate cyclase activity (Carson et al., 1996). In addition, during the preparation of this manuscript, Hurley et al. (1998) reported agonist-induced stimulation of [^{35}S]GTPγS binding and inhibition of adenylate cyclase activity for the human 5-ht$_{5A}$ receptor, expressed in HEK 293 cells.

In the present study, we have extensively investigated the binding properties, G protein coupling and activation of second messenger responses of the human (h) 5-ht$_{5A}$ receptor expressed in stably transfected human embryonic kidney (HEK) 293 (h5-ht$_{5A}$-HEK 293) cells. We have determined the effect of receptor-G protein uncoupling on high-affinity agonist ([^{3}H]5-CT) binding, by the addition of Gpp(NH)p and pre-treatment with pertussis toxin. We have investigated h5-ht$_{5A}$ receptor-mediated activation of G proteins, using agonist-stimulated [^{35}S]GTPγS binding and we have explored 5-HT-induced effects on cyclic adenosine monophosphate (cAMP) formation in h5-ht$_{5A}$-HEK 293 cells.

2. Materials and methods

2.1. Materials

The h5-ht$_{5A}$-HEK 293 cell line, stably expressing the human 5-ht$_{5A}$ receptor, was obtained from BioResearch Ireland (Dublin, Ireland). 5-Carboxamido[^{3}H]tryptamine ([^{3}H]5-CT, 50–100 Ci/mmol), [*N*-methyl-^{3}H]lysergic acid diethylamide ([^{3}H]LSD, 60–86 Ci/mmol) and guanosine-5′[γ-^{35}S]thiotriphosphate ([^{35}S]GTPγS, > 1000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). [2,8-^{3}H]Adenosine 3′,5′-cyclic phosphate ([^{3}H]cAMP, 25–40 Ci/mmol) and [2,8-^{3}H]adenine ([^{3}H]adenine), 20–40 Ci/mmol) were obtained from New England Nuclear Life Science Products (Boston, MA, USA). 5-HT, 5-methoxytryptamine (5-MT) and dihydroergotamine were from Acros Organics (Geel, Belgium); lysergic acid diethylamide (LSD) was from Kenija Industriji (Yugoslavia), 5-carboxamidotryptamine (5-CT) from Research Biochemicals International (Natick, MA, USA) and methiothepin from Hoffman-LaRoche (Basel, Switzerland). Dopamine, norepinephrine, pargyline and forskolin were from Sigma-Aldrich (St. Louis, MO, USA). 3-Isobutyl-1-methylxanthine was from Fluka (Buchs, Switzerland). Dulbecco's modified Eagle's medium and dialysed calf serum were obtained from Life Technologies (Gaithersburg, MD, USA). Pertussis toxin was obtained from Calbiochem (La Jolla, CA, USA). The protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Guanylyl-imidodiphosphate (Gpp(NH)p), GDP and GTPγS were from Boehringer-Mannheim (Mannheim, Germany). GF/B glass-fibre filters were from Whatman (Kent, UK). The Brandel 96-sample harvester was purchased from Brandel (Gaithersburg, MD, USA); the liquid scintillation spectrometer (Tricarb) and the scintillation fluid (Ultima Gold MV) were from Packard (Meriden, CT, USA). The GraphPad Prism program was from GraphPad Software, (San Diego, CA, USA).

5-HT, 5-CT, 5-MT, dopamine and norepinephrine were dissolved and diluted in assay buffer. Dihydroergotamine, LSD and methiothepin were dissolved and diluted in dimethylsulfoxide; the last 20-fold dilution step was performed in assay buffer. The dilution in the assay mixture was 10-fold. In all control assays, dimethylsulfoxide was added to a final concentration of 0.5%.

2.2. Cell culture and pertussis toxin treatment

Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated (30 min

at 56°C) dialysed calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Sodium butyrate (5 mM) was added 24 h before the assay to enhance receptor expression.

Pertussis toxin treatment of cells consisted of the addition of 100 ng pertussis toxin per ml growth medium, 24 h before harvesting. Untreated cells were grown in parallel.

2.3. Membrane preparation

Cells were cultured to 90% confluence on 150 mm petri dishes, washed with ice-cold phosphate-buffered saline, scraped from the plates in 50 mM Tris–HCl buffer, pH 7.4, and collected through centrifugation (10 min at 1000 $\times g$ at 4°C). The cell pellet was resuspended in hypotonic 10 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.05 mM benzamidine and 6 μg/ml leupeptin, kept on ice for 1 h and homogenised with a Dual homogeniser. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C. The resulting pellet was resuspended in the same buffer and again centrifuged at $1300 \times g$ for 10 min at 4°C. Membranes were collected from the pooled supernatants by centrifugation at $50{,}000 \times g$ for 1 h at 4°C. The membrane pellet was resuspended in 25 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 10% glycerol, 0.05 mM benzamidine and 6 μg/ml leupeptin and stored in aliquots at −80°C.

2.4. Determination of membrane protein content

Protein content in membrane preparations was estimated with the Bradford protein assay (Bradford, 1976), using the Bio-Rad kit. Bovine serum albumin was used as a standard.

2.5. Radioligand binding assays

For binding studies with [^{3}H]5-CT or [^{3}H]LSD, membranes were thawed on ice and diluted in 50 mM Tris–HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, 1 mM EGTA and 10 μM pargyline. Non-specific binding of the radioligand was estimated in the presence of 10 μM methiothepin. Assay mixtures were incubated for 1 h at 25°C in a volume of 0.5 ml containing approximately 6 μg of membrane protein. Reactions were terminated by rapid filtration through GF/B filters, pre-soaked in 0.1% polyethyleneimine, using a Brandel 96-sample harvester. Filters were then washed twice with 5 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4. Filter-bound radioactivity was counted in a liquid scintillation spectrometer, using 3 ml of scintillation fluid.

For radioligand concentration-binding isotherms, ten to sixteen concentrations of [^{3}H]5-CT, in a range of 0.1 nM to 25 nM, and ten to twelve concentrations of [^{3}H]LSD, in a range of 0.1 nM to 12 nM, were used. Competition binding experiments were performed using 2 nM [^{3}H]5-CT; compounds were added at twelve concentrations, spanning three orders of magnitude around the IC_{50} value.

Radioligand concentration-binding isotherms (rectangular hyperbola) and sigmoidal inhibition curves were calculated by non-linear regression analysis according to algorithms described by Oestreicher and Pinto (1987). The maximal number of binding sites (B_{max}), the apparent equilibrium dissociation constant (K_d) values of the radioligand and the IC_{50} (concentration that inhibits 50% of specific radioligand binding) values of the inhibitors were derived from the curve fitting. Figures were prepared using the GraphPad Prism program.

2.6. [^{35}S]GTPγS binding assays

In preliminary [^{35}S]GTPγS binding experiments, assay conditions were optimised by sequentially varying the amount of membrane protein (5 to 40 μg per assay), the NaCl concentration (0 to 150 mM), the GDP concentration (10 nM to 100 μM), the $MgCl_2$ concentration (0 to 100 mM) and the incubation time (5 min to 4 h). The following conditions resulted in optimal [^{35}S]GTPγS binding: membranes were thawed on ice and diluted in 50 mM Tris–HCl buffer, pH 7.4, containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM dithiothreitol, 10 μM pargyline and 1 μM GDP. Assay mixtures of 0.45 ml contained about 15 μg of membrane protein and were pre-incubated with compounds for 30 min at 30°C. Then, 50 μl of [^{35}S]GTPγS in assay buffer was added to a final concentration of 0.2 nM and the assay mixtures were further incubated for 30 min at 30°C. Reactions were terminated by rapid filtration through GF/B filters, pre-soaked in assay buffer, using a 40-well manual filtration manifold. Filters were washed twice with 5 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM EGTA. Filter-bound radioactivity was counted in a liquid scintillation spectrometer, using 3 ml of scintillation fluid. Basal [^{35}S]GTPγS binding was measured in the absence of compounds. Compounds were added at six to eight concentrations. In initial experiments, non-specific [^{35}S]GTPγS binding was measured in the presence of 100 μM GTPγS; it did never exceed 10% of basal binding and was never subtracted from experimental data.

Stimulation of [^{35}S]GTPγS binding was presented as percentage over basal and was calculated as hundred times the difference between stimulated and basal binding (in cpm), divided by the amount of basal binding (in cpm). Agonist concentration–response curves for increases in [^{35}S]GTPγS binding and antagonist inhibition curves for inhibition of 5-HT (10 μM)-stimulated [^{35}S]GTPγS binding were analysed by non-linear regression using the GraphPad Prism program. EC_{50} (concentration of com-

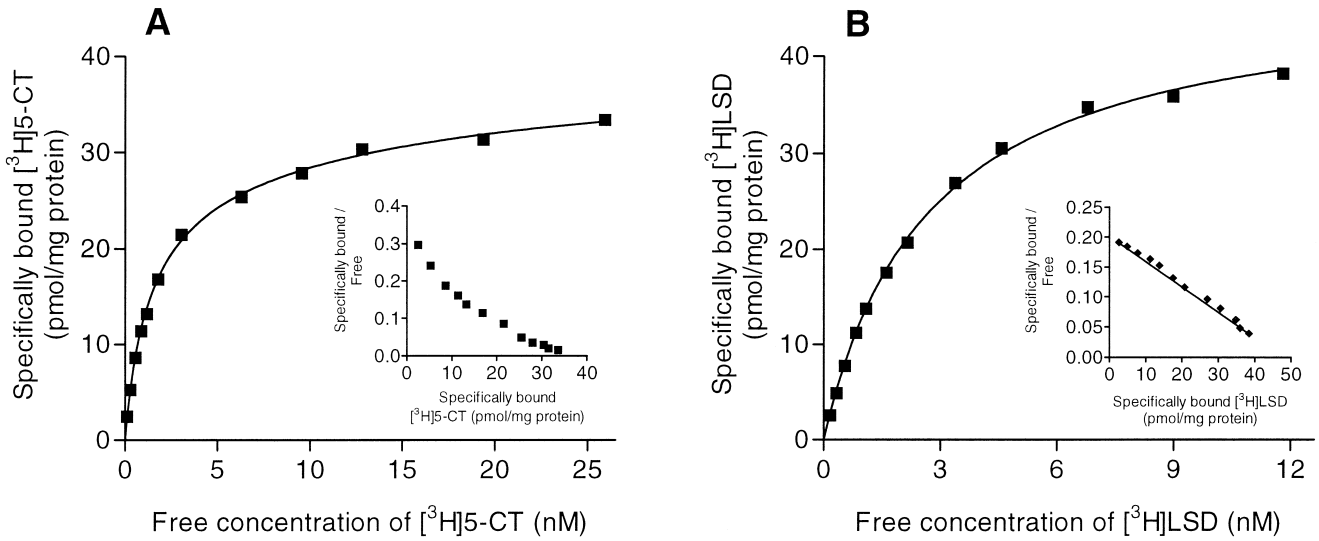

Fig. 1. Concentration-binding isotherms and Scatchard plots (insets) of [^{3}H]5-CT (Panel A) and [^{3}H]LSD (Panel B) binding to membranes of h5-ht$_{5A}$-HEK 293 cells. Membranes were incubated with a range of [^{3}H]5-CT or [^{3}H]LSD concentrations for 1 h at 25°C. Non-specific binding was determined in the presence of 10 μM methiothepin. Specific binding is presented as means of duplicate determinations. The data represent a typical experiment out of five ([^{3}H]LSD) or six ([^{3}H]5-CT) independent experiments. [^{3}H]5-CT concentration-binding isotherms were best fitted to a two-binding-site model using non-linear regression analysis, and [^{3}H]LSD concentration-binding isotherms were best fitted to a one-binding-site model. B_{max} and K_d values were derived for each individual experiment and mean values are summarised in Table 1.

pound at which 50% of its own maximal stimulation is obtained) and IC_{50} (concentration of compound at which 50% of its own maximal inhibition of 5-HT-stimulated [^{35}S]GTPγS binding is obtained) values were derived from the curves. IC_{50} values were corrected as follows: corrected IC_{50} (IC_{50}corr) = $IC_{50}/(1 + [5\text{-HT}]/EC_{50}(5\text{-HT}))$. Maximal stimulation ($E_{max}$) values were calculated as percentage of the maximal stimulation obtained with 10 μM 5-HT and maximal inhibition (I_{max}) values were calculated as percentage of the inhibition from maximal 5-HT (10 μM)-stimulated [^{35}S]GTPγS binding to basal level.

2.7. cAMP accumulation assays

cAMP formation was measured by loading of the cells with [^{3}H]adenine and isolation of [^{3}H]cAMP using column chromatography, based on the method described by Salomon (1997). Cells were plated into 24-well culture dishes 48 h before the assay at a cell density of 1×10^5 cells/well

Table 1
Effect of Gpp(NH)p addition and pertussis toxin pre-treatment on K_d and B_{max} values measured with [^{3}H]5-CT and [^{3}H]LSD for binding to membranes of h5-ht$_{5A}$-HEK 293 cells

Radioligand	PTX treatment[a]	Gpp(NH)p addition	K_d (nM)	B_{max} (pmol/mg protein)	n
[^{3}H]5-CT	Untreated	no addition	2.3 ± 0.3[b]	31 ± 2[b]	6
		High	0.4 ± 0.1	8 ± 2	
		Low	5.5 ± 0.9	25 ± 2	
		Gpp(NH)p added	4.3 ± 0.5	29 ± 4	4
	PTX-treated[a]	no addition	3.8 ± 0.3	32 ± 1	6
		Gpp(NH)p added	4.1 ± 0.3	31 ± 3	4
[^{3}H]LSD	Untreated	no addition	2.3 ± 0.1	49 ± 1	5
		Gpp(NH)p added	2.4 ± 0.2	49 ± 1	3
	PTX-treated[a]	no addition	2.6 ± 0.2	47 ± 2	5
		Gpp(NH)p added	2.3 ± 0.3	48 ± 3	3

Radioligand binding studies were performed and K_d and B_{max} values were derived as described in Section 2. The results are means ± S.E.M. from three to six (n) independent experiments. 'High' and 'Low' represent the K_d and B_{max} values of high- and low-affinity agonist binding site, respectively.
[a] PTX, Pertussis toxin.
[b] Values obtained from curve-fitting to a one-binding-site model, although a significantly better fit was achieved with a two-binding-site model (GraphPad Prism program, F test, $P < 0.005$).

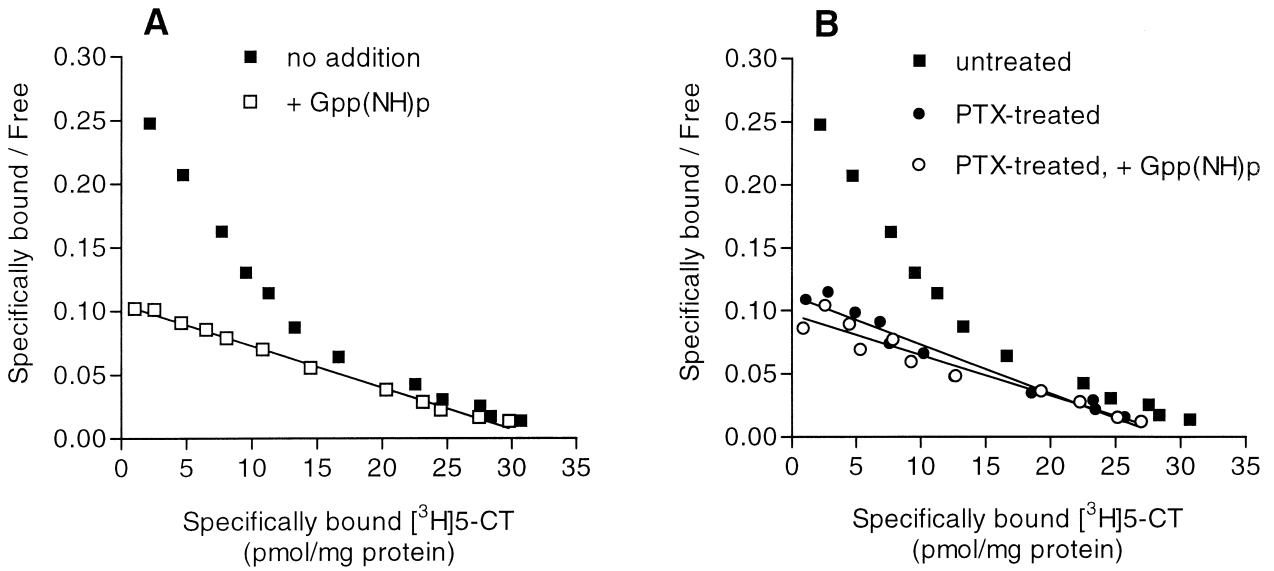

Fig. 2. Effects of Gpp(NH)p addition and pertussis toxin pre-treatment on [^{3}H]5-CT binding to membranes of h5-ht$_{5A}$-HEK 293 cells. Panel A. Scatchard plot of concentration-binding isotherms of specific [^{3}H]5-CT binding to membranes of h5-ht$_{5A}$-HEK 293 cells in the absence (■) or presence (□) of 100 μM Gpp(NH)p. Panel B. Scatchard plot of concentration-binding isotherms of specific [^{3}H]5-CT binding to membranes of untreated (■) and pertussis toxin-treated h5-ht$_{5A}$-HEK 293 cells in the absence (●) or presence (○) of 100 μM Gpp(NH)p. Pertussis toxin was added at 100 ng/ml culture medium, 24 h before membrane preparation. Untreated h5-ht$_{5A}$-HEK 293 cells were grown in parallel. Radioligand binding studies were performed as described under Section 2. The data represent a typical experiment out of four (Gpp(NH)p added) or six (no addition) independent experiments. Mean K_d and B_{max} values are given in Table 1. PTX, Pertussis toxin.

and were treated with sodium butyrate (5 mM) after 24 h. Cells were washed with controlled salt solution (CSS; 120 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, 0.04 mM phenol red in 25 mM Tris–HCl, pH 7.4) and incubated for 2 h at 37°C with 1.5 μCi [^{3}H]adenine in CSS. Cells were then washed twice with CSS and incubated at 37°C for 20 min in 0.5 ml CSS, containing 1 mM 3-isobutyl-1-methylxanthine, 1 μM pargyline and 1 μM paroxetine, supplemented with 100 μM forskolin and/or defined concentrations of 5-HT. The incubation was terminated by addition of 0.1 ml of ice-cold $HClO_4$ (1 N), followed by neutralisation to pH 7.4 with 0.1 ml of ice-cold KOH/K_3PO_4 (0.5 M), pH 13.5. Following $KClO_4$ precipitation, plates were centrifuged for 5 min at $650 \times g$ (at 4°C) and 0.5 ml of each supernatant was applied to a cation exchange column (2 ml of Dowex 50W-X4, 200–400 mesh). The column was washed with 2.5 ml MilliQ water and then positioned over a column of aluminium oxide (1 g) that was previously saturated with 0.1 M imidazole–HCl, pH 7.4. [^{3}H]cAMP was eluted from the Dowex column with 3 ml of MilliQ water and eluted from the aluminium oxide column with 2 ml of 0.1 M imidazole, pH 7.4. This fraction was collected and the radioactivity was measured, using a liquid scintillation spectrometer, to determine the amount of accumulated [^{3}H]cAMP. The recovery of cAMP, as measured in three separate experiments using a [^{3}H]cAMP standard, was 51 ± 2% (mean ± S.E.M.).

cAMP levels were expressed as percentage of the forskolin-stimulated level in the absence of 5-HT and plotted against the concentration of 5-HT on a log scale. Sigmoidal curves were fitted by non-linear regression analysis and IC_{50} (concentration of 5-HT at which 50% of its own maximal inhibition of forskolin-stimulated cAMP level

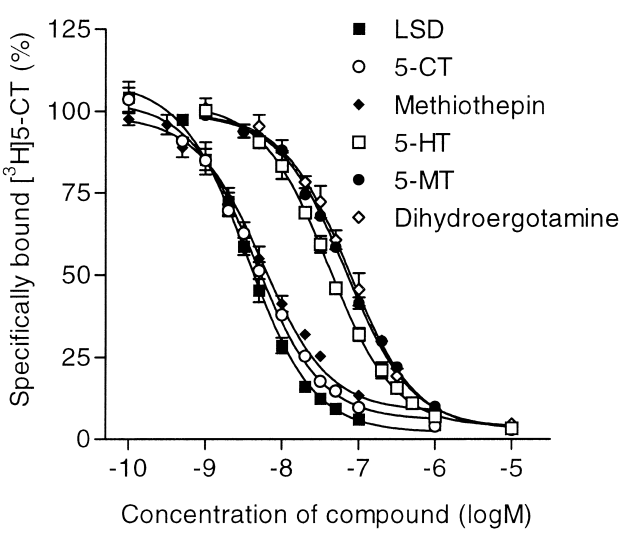

Fig. 3. Inhibition of [^{3}H]5-CT binding to membranes of h5-ht$_{5A}$-HEK 293 cells by 5-HT receptor ligands. Membranes were incubated for 1 h at 25°C with 2 nM of [^{3}H]5-CT and concentrations of compounds ranging from 0.1 nM to 10 μM. Non-specific binding was determined in the presence of 10 μM methiothepin. Depicted points are means ± S.E.M. of three to seven independent experiments. For each individual experiment the pIC_{50} value was derived from curve fitting using non-linear regression analysis. Mean pIC_{50} values are given in Table 2.

Table 2
Inhibition by various 5-HT receptor ligands of [^{3}H]5-CT binding to membranes of h5-ht$_{5A}$-HEK 293 cells

Compound	pIC$_{50}$	*n*
LSD	8.40 ± 0.03	4
5-CT	8.28 ± 0.03	4
Methiothepin	8.26 ± 0.07	4
5-HT	7.40 ± 0.03	7
5-MT	7.17 ± 0.06	3
Dihydroergotamine	7.15 ± 0.05	4
Dopamine	4.91 ± 0.05	3
Norepinephrine	3.72 ± 0.07	3

Radioligand binding studies were performed as described under Section 2 and pIC$_{50}$ values were derived from individual curves. The results are mean pIC$_{50}$ values ± S.E.M. from three to seven (*n*) independent experiments. Mean inhibition curves are shown in Fig. 3.

is obtained) values were derived using the GraphPad Prism program.

3. Results

3.1. Binding of [^{3}H]5-CT and [^{3}H]LSD to h5-ht$_{5A}$-HEK 293 cell membranes

The h5-ht$_{5A}$ receptor binding properties were investigated using [^{3}H]5-CT and [^{3}H]LSD binding to membranes of h5-ht$_{5A}$-HEK 293 cells. The [^{3}H]5-CT concentration-binding isotherms were best fitted to a two-binding-site model using non-linear regression (GraphPad Prism program, *F* test, $P < 0.005$) (Fig. 1-A). The biphasic Scatchard plot, shown in Fig. 1-A (inset), clearly demonstrated the presence of both high- and low-affinity sites. Mean K_d and B_{max} values from curve fitting to a one-binding-site and a two-binding-site model are given in Table 1 ('untreated, no addition'). The difference in affinity between the high- and low-affinity [^{3}H]5-CT binding sites was about 14-fold and approximately 25% of the receptor population was present in the high-affinity binding state. Membrane preparations of h5-ht$_{5A}$-HEK 293 cells expressing fewer h5-ht$_{5A}$ receptors displayed a higher percentage of high-affinity receptors. For example, in membranes with a total of 25 pmol [^{3}H]5-CT binding sites per mg of protein, about 35% of the receptors were in the high-affinity state (data not shown). [^{3}H]LSD bound to a single, saturable site (Fig. 1-B; Table 1). Compared to [^{3}H]5-CT, [^{3}H]LSD labelled about 40% more binding sites.

3.2. Effects of Gpp(NH)p and pertussis toxin on [^{3}H]5-CT and [^{3}H]LSD binding to h5-ht$_{5A}$-HEK 293 cell membranes

To investigate interactions of the h5-ht$_{5A}$ receptor with cellular G proteins, the effect on [^{3}H]5-CT and [^{3}H]LSD binding of addition of the non-hydrolysable GTP analogue

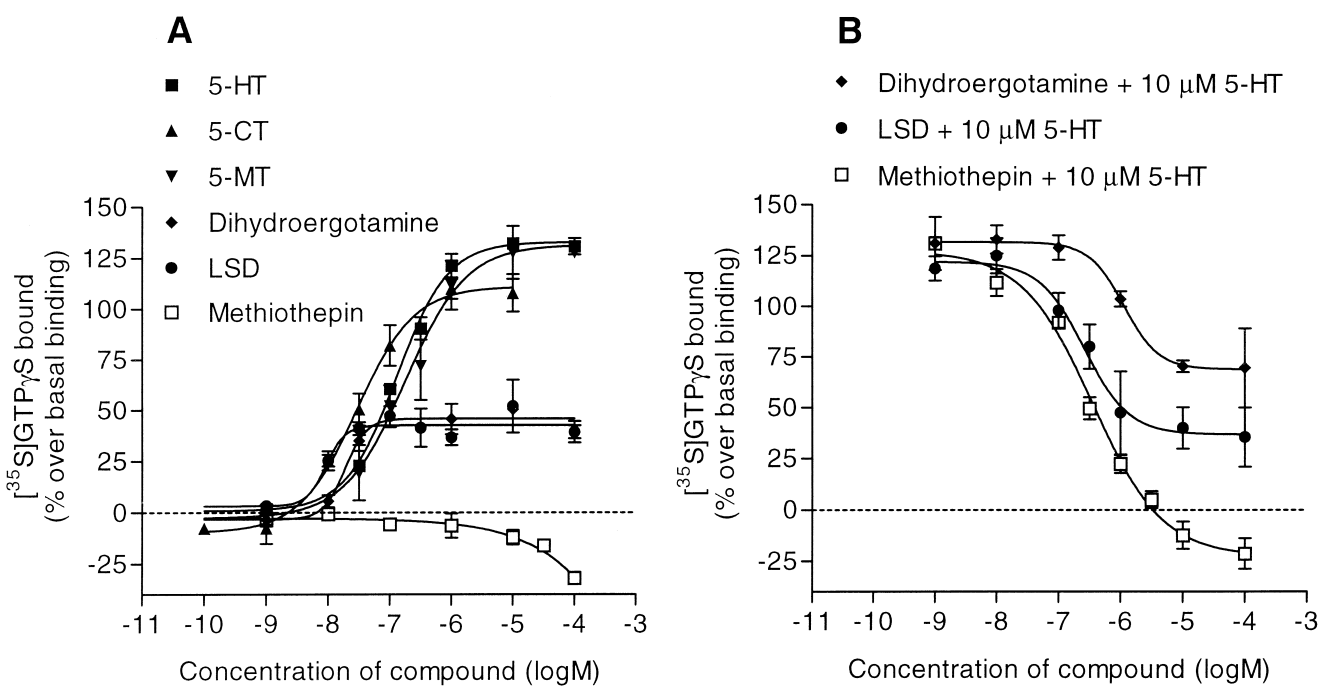

Fig. 4. [^{35}S]GTPγS binding to membranes of h5-ht$_{5A}$-HEK 293 cells. Panel A. Stimulation of [^{35}S]GTPγS binding to h5-ht$_{5A}$-HEK 293 cell membranes by 5-HT receptor agonists. Panel B. Antagonism of 5-HT (10 μM)-stimulated [^{35}S]GTPγS binding to h5-ht$_{5A}$-HEK 293 cell membranes by 5-HT receptor ligands. Membranes were pre-incubated with compounds for 30 min at 30°C and incubated with 0.2 nM [^{35}S]GTPγS for a further 30 min at 30°C. Basal [^{35}S]GTPγS binding was measured in the absence of compounds and the percentage of stimulation was calculated as defined under Section 2. Depicted points are mean values ± S.E.M. from three independent experiments, each performed in duplicate. Mean pEC$_{50}$, E_{max}, corrected pIC$_{50}$ (pIC$_{50}$corr) and I_{max} values are summarised in Table 3.

Gpp(NH)p to membranes and the effect of pertussis toxin pre-treatment of h5-ht$_{5A}$-HEK 293 cells were determined. Derived K_d and B_{max} values are shown in Table 1. Addition of 100 μM Gpp(NH)p abolished the high-affinity binding of [^{3}H]5-CT. A single, saturable low-affinity binding site was detected, as shown in the Scatchard plot (Fig. 2-A). Pertussis toxin pre-treatment of h5-ht$_{5A}$-HEK 293 cells also abolished high-affinity binding of [^{3}H]5-CT, as shown in Fig. 2-B. Both Gpp(NH)p and pertussis toxin shifted the K_d of [^{3}H]5-CT to the same extent; the low-affinity K_d value was reached. Addition of Gpp(NH)p to membranes of pertussis toxin-treated cells did not further decrease the affinity of [^{3}H]5-CT (Fig. 2-B). In contrast to [^{3}H]5-CT, the affinity of [^{3}H]LSD was not clearly affected by Gpp(NH)p addition or pertussis toxin pre-treatment (Table 1).

3.3. Inhibition of [^{3}H]5-CT binding by various compounds

The pharmacological profile of the h5-ht$_{5A}$ receptor expressed in HEK 293 cells was determined by analysing inhibition of [^{3}H]5-CT binding by various 5-HT receptor ligands (Fig. 3). pIC$_{50}$ ($-\log$ IC$_{50}$) values were derived from non-linear regression analysis and mean values are shown in Table 2. The rank order of potency of the tested compounds was: LSD ≥ 5-CT ≈ methiothepin > 5-HT ≥ 5-MT ≈ dihydroergotamine >> dopamine > norepinephrine.

3.4. Agonist-stimulated [^{35}S]GTPγS binding

Several 5-HT receptor ligands were examined for their ability to promote receptor-mediated G protein activation, using the stimulation of [^{35}S]GTPγS binding to membranes of h5-ht$_{5A}$-HEK 293 cells (Fig. 4-A). pEC$_{50}$ and E_{max} values were determined and are summarised in Table 3.

5-HT stimulated binding of [^{35}S]GTPγS to membranes of h5-ht$_{5A}$-HEK 293 cells with a maximal response of about 130% over the basal level. The 5-HT effect on [^{35}S]GTPγS binding was specific for the h5-ht$_{5A}$ receptor, as 5-HT did not affect [^{35}S]GTPγS binding to membrane preparations of non-transfected HEK 293 cells (data not shown). 5-MT and 5-CT produced maximal responses similar to 5-HT, qualifying these compounds as full agonists. 5-MT displayed a similar potency as 5-HT, while 5-CT had a higher potency. LSD and dihydroergotamine also had higher potencies than 5-HT, but behaved as partial agonists, with maximal stimulation values of 46% and 43% over the basal level, respectively. Methiothepin behaved as an inverse agonist, as it inhibited basal [^{35}S]GTPγS binding to h5-ht$_{5A}$-HEK 293 cell membranes at concentrations ≥ 10 μM. However, no plateau in maximal inhibition (about 25%) was detected at 100 μM.

Table 3
Effect of various 5-HT receptor ligands on [^{35}S]GTPγS binding to membranes of h5-ht$_{5A}$-HEK 293 cells

Compound	pEC$_{50}$	E_{max} (%)
5-HT	6.95 ± 0.04	100 ± 2
5-CT	7.48 ± 0.10	82 ± 4
5-MT	6.79 ± 0.07	96 ± 9
Dihydroergotamine	7.63 ± 0.09	38 ± 2
LSD	8.28 ± 0.04	33 ± 4
	pIC$_{50}$corr	I_{max} (%)
Dihydroergotamine	7.43 ± 0.04	49 ± 6
LSD	8.54 ± 0.19	74 ± 10
Methiothepin	8.47 ± 0.10	115 ± 5

[^{35}S]GTPγS binding experiments were performed as described in Section 2 and pEC$_{50}$, E_{max}, pIC$_{50}$ and I_{max} values were derived from the curves. pIC$_{50}$ values were corrected into pIC$_{50}$corr values as described in Section 2. The results are means ± S.E.M. from three independent experiments.

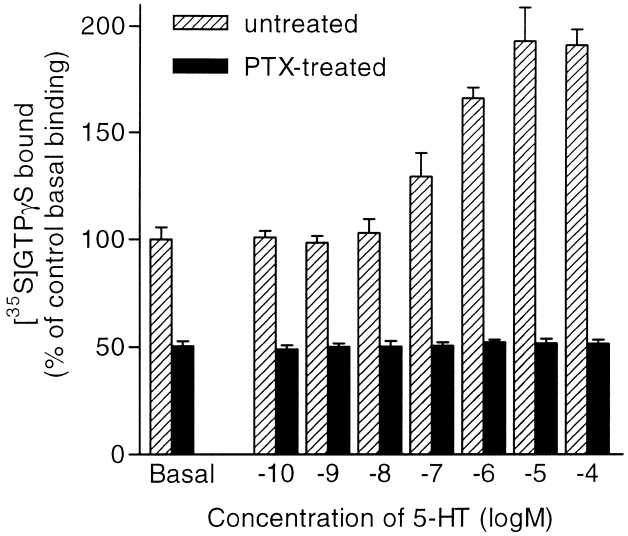

Fig. 5. Effect of pertussis toxin pre-treatment on the 5-HT-mediated stimulation of [^{35}S]GTPγS binding to membranes of h5-ht$_{5A}$-HEK 293 cells. Pertussis toxin was added at 100 ng/ml culture medium, 24 h before membrane preparation. Untreated cells were grown in parallel. Membranes were pre-incubated with 5-HT for 30 min at 30°C and incubated with 0.2 nM [^{35}S]GTPγS for a further 30 min at 30°C. Basal [^{35}S]GTPγS binding was measured in the absence of compound. Depicted data are mean values ± S.E.M. from three independent experiments, each performed in duplicate. Data are expressed as percentage of the control basal level (basal [^{35}S]GTPγS binding to membranes from untreated cells). PTX, Pertussis toxin.

3.5. Antagonism of 5-HT-stimulated [^{35}S]GTPγS binding

5-HT receptor ligands were also examined for their ability to inhibit 5-HT (10 μM)-stimulated [^{35}S]GTPγS binding to membranes of h5-ht$_{5A}$-HEK 293 cells. 5-MT and 5-CT had no effect on maximal 5-HT-stimulated [^{35}S]GTPγS binding (data not shown). LSD, dihydroergotamine and methiothepin had antagonistic properties (Fig. 4-B); they inhibited the 5-HT-stimulated [^{35}S]GTPγS binding to the level of their own agonistic effect. Corrected pIC$_{50}$ and I_{max} values were determined and are summarised in Table 3. Methiothepin acted again as an inverse

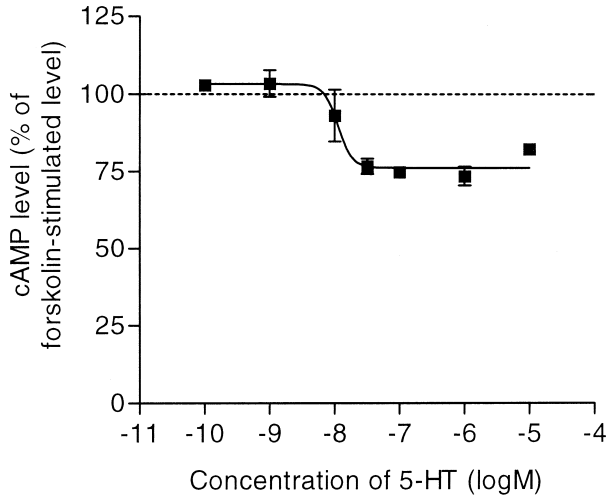

Fig. 6. Inhibition by 5-HT of forskolin-stimulated cAMP accumulation in h5-ht_{5A}-HEK 293 cells. Cells were plated into 24-well culture dishes 48 h before the assay and treated with sodium butyrate (5 mM) after 24 h. The cells were then loaded with [^{3}H]adenine for 2 h and the cAMP accumulation assay was performed as described under Section 2. Depicted points are means ± S.E.M. of three independent experiments, each performed in triplicate. The mean (± S.E.M.) of the individual pIC_{50} values, that were derived from the curve fitting using non-linear regression analysis, was 8.0 ± 0.2.

agonist, as it inhibited 5-HT-stimulated [^{35}S]GTPγS binding to 15% below basal level.

3.6. Effect of pertussis toxin pre-treatment on 5-HT-stimulated [^{35}S]GTPγS binding

The 5-HT-mediated stimulation of [^{35}S]GTPγS binding to membranes of h5-ht_{5A}-HEK 293 cells was completely abolished by pre-treatment of the cells with 100 ng/ml pertussis toxin for 24 h (Fig. 5). Basal binding of [^{35}S]GTPγS was decreased by 50% and no change in this level was observed even in the presence of 100 μM 5-HT.

3.7. 5-HT-stimulated inhibition of cAMP accumulation

The effect of 5-HT on cAMP accumulation in h5-ht_{5A}-HEK 293 cells is shown in Fig. 6. 5-HT caused a concentration-dependent inhibition of cAMP formation to about 25% below the forskolin (100 μM)-stimulated level and with a pIC_{50} of 8.0 ± 0.2 ($n = 3$). Treatment of h5-ht_{5A}-HEK 293 cells with 5-HT did not affect basal cAMP content (data not shown). Attempts to demonstrate 5-HT-induced effects on arachidonic acid release and changes in the intracellular Ca^{2+} concentration in h5-ht_{5A}-HEK 293 cells were unsuccessful (data not shown).

4. Discussion

We have investigated the binding and the G protein coupling properties of the h5-ht_{5A} receptor expressed in stably transfected HEK 293 cells and have demonstrated h5-ht_{5A} receptor-mediated G protein activation and inhibition of adenylate cyclase. Previously reported expression studies in COS cells failed to demonstrate coupling of the h5-ht_{5A} receptor to G proteins (Rees et al., 1994; Grailhe et al., 1995). During the preparation of this manuscript, however, functional coupling to G proteins was reported for the h5-ht_{5A} receptor expressed in HEK 293 cells (Hurley et al., 1998). Our study confirms this finding and extends it with additional data from radioligand and [^{35}S]GTPγS binding experiments, in which various other 5-HT receptor ligands were tested and the effects of Gpp(NH)p addition to membranes and pertussis toxin pre-treatment of cells were determined.

4.1. [^{3}H]5-CT and [^{3}H]LSD binding to membranes of h5-ht_{5A}-HEK 293 cells

We performed concentration-binding experiments with [^{3}H]5-CT and [^{3}H]LSD on h5-ht_{5A}-HEK 293 cell membranes (Fig. 1, Table 1).

The agonist [^{3}H]5-CT displayed two distinct binding affinities, suggesting that a fraction of the h5-ht_{5A} receptors is present in a high-affinity, G protein-coupled form. Guanine nucleotides, such as GTP or Gpp(NH)p, have been shown to uncouple various G protein-coupled receptors (Lefkowitz et al., 1976). For the mouse 5-ht_{5A} receptor expressed in NIH 3T3 cells, inhibition of [^{3}H]LSD binding by agonists was shown to be GTP-sensitive (Plassat et al., 1992). Addition of Gpp(NH)p also reduced the fraction of rat 5-ht_{5B} receptors present in the high-affinity state, as determined by binding of [^{3}H]5-CT to membranes of transiently transfected COS-1 cells (Wisden et al., 1993). However, [^{3}H]5-CT binding to the h5-ht_{5A} receptor expressed in COS-7 cells was insensitive to Gpp(NH)p (Grailhe et al., 1995). To confirm that the presence of two sites with different affinities in membranes of h5-ht_{5A}-HEK 293 cells is indeed due to interaction of the receptor with G proteins, we investigated the effect of Gpp(NH)p on radioligand binding (Table 1, Fig. 2-A). Gpp(NH)p indeed converted the high-affinity [^{3}H]5-CT binding sites into low-affinity sites. This observation confirms the occurrence of h5-ht_{5A} receptor-G protein interaction in membranes of h5-ht_{5A}-HEK 293 cells. Pertussis toxin ADP-ribosylates G protein α_i, α_o and α_t subunits and thereby prevents receptors from interacting with and activating these G proteins. Pre-treatment of h5-ht_{5A}-HEK 293 cells with pertussis toxin abolished high-affinity [^{3}H]5-CT binding (Table 1, Fig. 2-B), indicating coupling of the h5-ht_{5A} receptor to pertussis toxin-sensitive G proteins. Since addition of Gpp(NH)p to membranes of pertussis toxin-treated cells did not further decrease the affinity of [^{3}H]5-CT, we conclude that all high-affinity sites represent receptors that couple to pertussis toxin-sensitive G proteins upon agonist binding.

In contrast to [^{3}H]5-CT, [^{3}H]LSD revealed only one binding affinity and labelled about 40% more sites than [^{3}H]5-CT. The affinity of [^{3}H]LSD was not affected by either Gpp(NH)p addition, pertussis toxin treatment or addition of Gpp(NH)p to membranes of pertussis toxin-treated cells (Table 1). These observations clearly show that LSD behaves as an antagonist in binding studies on the h5-ht$_{5A}$ receptor.

4.2. Pharmacological profile

We determined the pharmacological profile of the h5-ht$_{5A}$ receptor using inhibition of [^{3}H]5-CT binding by various 5-HT receptor ligands (Table 2, Fig. 3). Of the tested compounds, LSD displayed the highest affinity, which was comparable to the affinity of LSD for the mouse 5-ht$_{5A}$ (Plassat et al., 1992) and rat 5-ht$_{5B}$ receptor (Wisden et al., 1993). The affinity constant for methiothepin was comparable to the values reported previously (Rees et al., 1994; Hurley et al., 1998) and was between one and two orders of magnitude higher than for the mouse (Plassat et al., 1992) and rat 5-ht$_{5A}$ receptor (Erlander et al., 1993). 5-CT and 5-HT displayed affinities that were in good agreement with those reported by Hurley et al. (1998), but were substantially higher than for the receptor expressed in COS-M6 cells (Rees et al., 1994). This agonist affinity difference is probably due to interaction of the receptor with G proteins in the HEK 293 cell membranes, while apparently no receptor-G protein coupling was present in COS cells. 5-MT and dihydroergotamine displayed similar, rather low affinities for the h5-ht$_{5A}$ receptor.

4.3. Stimulation of [^{35}S]GTPγS binding

To directly examine activation of G proteins by agonist stimulation of the h5-ht$_{5A}$ receptor, we performed [^{35}S]GTPγS binding assays (Table 3, Fig. 4). Receptor-mediated G protein activation involves stimulation of nucleotide exchange (GDP for GTP) at the G_α subunit and can be measured by the incorporation of the non-hydrolysable GTP analogue [^{35}S]GTPγS (Wieland and Jakobs, 1994; Lazareno, 1997). The maximal stimulation of [^{35}S]GTPγS binding to h5-ht$_{5A}$-HEK 293 cell membranes obtained with tryptamine analogs was about 130% over basal and is nearly seven times larger than that reported by Hurley et al. (1998) for 5-CT. Both LSD and dihydroergotamine stimulated [^{35}S]GTPγS binding to a submaximal level, indicating that these compounds behave as partial agonists. These two compounds also exhibited antagonistic properties; their agonist and antagonist curves almost converged. Methiothepin behaved as an inverse agonist, reducing basal [^{35}S]GTPγS binding at high concentrations and antagonising the 5-HT-induced stimulation of [^{35}S]GTPγS binding below the basal level. The inverse agonistic effect of methiothepin in [^{35}S]GTPγS binding experiments has also been observed for 5-HT receptors of the 5-HT$_1$ subfamily: h5-HT$_{1A}$ (Stanton and Beer, 1997), h5-HT$_{1B}$ and h5-HT$_{1D}$ (Pauwels et al., 1997).

The enhancement of [^{35}S]GTPγS binding by 5-HT was completely blocked by pre-treatment of the h5-ht$_{5A}$-HEK 293 cells with pertussis toxin, confirming that the event is mediated entirely through pertussis toxin-sensitive G proteins (Fig. 5). Pertussis toxin treatment also lowered basal [^{35}S]GTPγS binding. This may imply that unoccupied receptors are actively coupled to pertussis toxin-sensitive G proteins and thus stimulate basal [^{35}S]GTPγS binding to G_α proteins. However, we can not rule out constitutive coupling to G_i/G_o proteins of other, endogenous receptors in HEK 293 cells. A similar reduction of basal [^{35}S]GTPγS binding by pertussis toxin treatment was also observed for other G protein-coupled receptors, e.g., μ-opioid receptors in SH-SY5Y cells (Traynor and Nahorski, 1995).

4.4. Inhibition of adenylate cyclase

Previously reported expression studies in COS cells did not reveal coupling of the h5-ht$_{5A}$ receptor to second messenger responses (Rees et al., 1994; Grailhe et al., 1995). Regarding 5-ht$_5$ receptors from other species, only the rat 5-ht$_{5A}$ receptor expressed in C6 glioma cells was reported to inhibit adenylate cyclase activity (Carson et al., 1996).

We determined the effect of 5-HT on arachidonic acid release, intracellular cAMP and intracellular Ca^{2+} concentrations in h5-ht$_{5A}$-HEK 293 cells to investigate h5-ht$_{5A}$ receptor-mediated modulation of second messenger systems. We did not find 5-HT-induced changes in intracellular Ca^{2+} concentration or arachidonic acid release, nor stimulation of cAMP formation (data not shown). However, we detected a concentration-dependent inhibition of cAMP formation by 5-HT, using a column method for the determination of the cellular [^{3}H]cAMP content (Fig. 6). 5-HT reduced the forskolin-stimulated cAMP accumulation by about 25% and with a pIC_{50} value of approximately 8.0. These data are comparable to those for 5-CT that were reported recently by Hurley et al. (1998). It is emphasised that the response could not be reproducibly shown by using a radio-immuno-assay for the quantification of accumulated cAMP in 96-well micro-titre plates, probably because of the small inhibitory effect. Why inhibition of adenylate cyclase activity was not detected in COS cells transfected with the h5-ht$_{5A}$ receptor (Rees et al., 1994; Grailhe et al., 1995) is unclear, but might be due to the apparent lack of receptor-G protein interaction in these cells. This lack of G protein coupling could be explained by the absence in COS cells of particular G protein subtypes, that are present in HEK 293 cells. Alternatively, differences in culture medium composition between the various studies could provide an explanation. COS cells were cultured in medium supplemented with 10% foetal calf serum (Rees et al., 1994), which can

contain a substantial concentration of 5-HT, while we have used 10% dialysed calf serum, which is depleted of 5-HT. We have determined the concentration of 5-HT in both media by High Performance Liquid Chromatography (data not shown). In medium with 10% dialysed calf serum, the 5-HT concentration was below 1 nM, while medium with 10% foetal calf serum contained 5-HT concentrations in the low μM range. The permanent exposure to 5-HT of cells cultured in medium containing non-dialysed serum might cause desensitisation of the receptor and result in uncoupling of the receptor from G proteins.

In this study, we have demonstrated h5-ht$_{5A}$ receptor-mediated functional responses, namely agonist-stimulated [^{35}S]GTPγS binding and inhibition of adenylate cyclase activity, in stably transfected HEK 293 cells. Others have provided evidence for the presence of 5-ht$_{5A}$ and 5-ht$_{5B}$ receptors in the brain by mRNA localisation studies, allowing the formulation of possible physiological roles of the receptor (Plassat et al., 1992; Erlander et al., 1993; Matthes et al., 1993; Wisden et al., 1993; Rees et al., 1994; Carson et al., 1996; Pasqualetti et al., 1998). In addition, Carson et al. (1996) have demonstrated 5-ht$_{5A}$ receptor protein in the rat brain, using immunohistochemistry. Furthermore, in vitro receptor autoradiography, using [^{125}I]LSD in the presence of masking compounds to block other receptor sites, has revealed the presence of specific 5-ht$_{5A}$ receptor sites in wild-type mice, while these sites were absent in knockout mice lacking a functional 5-ht$_{5A}$ gene (Waeber et al., 1997). Interestingly, in the presence of Gpp(NH)p, 5-CT displaced [^{125}I]LSD binding with a 6-fold lower affinity, suggesting coupling of the 5-ht$_{5A}$ receptor to G proteins in the mouse brain (Waeber et al., 1997; Hen R., communication at the *Fourth IUPHAR Satellite Meeting on Serotonin*, Rotterdam, The Netherlands, July 23–25, 1998). Taken together, these data strongly suggest the presence of a functionally active receptor in the brain. Therefore, we would propose to change the lower case NC-IUPHAR nomenclature, 5-ht$_{5A}$, into an upper case appellation, 5-HT$_{5A}$.

In conclusion, we found functional coupling of the h5-ht$_{5A}$ receptor to G_i/G_o proteins and receptor-mediated inhibition of adenylate cyclase activity in h5-ht$_{5A}$-HEK 293 cells. Agonist radioligand binding and 5-HT-stimulated [^{35}S]GTPγS binding to membranes of h5-ht$_{5A}$-HEK 293 cells were shown to be sensitive to Gpp(NH)p and pertussis toxin and cAMP formation was inhibited upon h5-ht$_{5A}$ receptor activation by 5-HT.

Acknowledgements

This work was supported by a grant from the IWT (Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie). The scientific advice of Katty Josson is much appreciated. We thank Walter Gommeren, Paul Van Gompel, Ria Wouters, Danielle van de Wiel and Martine Ercken for their helpful practical advice.

References

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochem. 72, 248–254.

Branchek, T.A., Zgombick, J.M., 1997. Molecular biology and potential functional role of 5-HT$_5$, 5-HT$_6$, and 5-HT$_7$ receptors. In: Baumgarten, H.G., Göthert, M. (Eds.), Handbook of experimental pharmacology. Serotonergic neurons and 5-HT receptors in the CNS. Springer-Verlag, Berlin, pp. 475–497.

Carson, M.J., Thomas, E.A., Danielson, P.E., Sutcliffe, J.G., 1996. The 5-HT$_{5A}$ serotonin receptor is expressed predominantly by astrocytes in which it inhibits cAMP accumulation: a mechanism for neuronal suppression of reactive astrocytes. Glia 17, 317–326.

Erlander, M.G., Lovenberg, T.W., Baron, B.M., de Lecea, L., Danielson, P.E., Racke, M., Slone, A.L., Siegel, B.W., Foye, P.E., Cannon, K., Burns, J.E., Sutcliffe, J.G., 1993. Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. Proc. Natl. Acad. Sci. USA 90, 3452–3456.

Grailhe, R., Ramboz, S., Boschert, U., Hen, R., 1995. The 5-HT$_5$ receptors: characterization of the human 5-HT$_{5A}$ receptor; absence of the human 5-HT$_{5B}$ receptor; knockout of the mouse 5-HT$_{5A}$ receptor. Soc. Neurosci. Abstr. 21, 1856.

Grailhe, R., Waeber, C., Brunner, D., Zhuang, X., Huang, Y., Moskowitz, M.A., Hen, R., 1997. The 5-HT$_{5A}$ receptors: localization in the CNS and increased exploratory activity in 5-HT$_{5A}$ knockout mice. Soc. Neurosci. Abstr. 23, 1216.

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

Humphrey, P.P.A., 1997. The characterization and classification of neurotransmitter receptors. Ann. New York Acad. Sci. 812, 1–13.

Hurley, P.T., McMahon, R.A., Fanning, P., O'Boyle, K.M., Rogers, M., Martin, F., 1998. Functional coupling of a recombinant human 5-HT$_{5A}$ receptor to G-proteins in HEK-293 cells. Br. J. Pharmacol. 124, 1238–1244.

Lazareno, S., 1997. Measurement of agonist-stimulated [^{35}S]GTPγS binding to cell membranes. Methods Mol. Biol. 83, 107–116.

Lefkowitz, R.J., Mullikin, D., Caron, M.G., 1976. Regulation of beta-adrenergic receptors by guanyl-5′-yl imidodiphosphate and other purine nucleotides. J. Biol. Chem. 251, 4686–4692.

Matthes, H., Boschert, U., Amlaiky, N., Grailhe, R., Plassat, J.-P., Muscatelli, F., Mattei, M.G., Hen, R., 1993. Mouse 5-hydroxytryptamine$_{5A}$ and 5-hydroxytryptamine$_{5B}$ receptors define a new family of serotonin receptors: cloning, functional expression, and chromosomal localization. Mol. Pharmacol. 43, 313–319.

Oestreicher, E.G., Pinto, G.F., 1987. A microcomputer program for fitting enzyme inhibition equations. Comput. Biol. Med. 17, 53–68.

Pasqualetti, M., Ori, M., Nardi, I., Castagna, M., Cassano, G.B., Marazziti, D., 1998. Distribution of the 5-HT$_{5A}$ serotonin receptor mRNA in the human brain. Mol. Brain Res. 56, 1–8.

Pauwels, P.J., Tardif, S., Palmier, C., Wurch, T., Colpaert, F.C., 1997. How efficacious are 5-HT$_{1B/D}$ receptor ligands: an answer from GTPγS binding studies with stably transfected C6-glial cell lines. Neuropharmacology 36, 499–512.

Plassat, J.-L., Boschert, U., Amlaiky, N., Hen, R., 1992. The mouse 5-HT$_5$ receptor reveals a remarkable heterogeneity within the 5-HT$_{1D}$ receptor family. EMBO J. 11, 4779–4786.

Rees, S., den Daas, I., Foord, S., Goodson, S., Bull, D., Kilpatrick, G., Lee, M., 1994. Cloning and characterisation of the human 5-HT$_{5A}$ serotonin receptor. FEBS Lett. 355, 242–246.

Salomon, Y., 1997. Cellular responsiveness to hormones and neurotrans-

mitters: conversion of [^{3}H]adenine to [^{3}H]cAMP in cell monolayers, cell suspensions and tissue slices. Methods Enzymol. 195, 22–28.

Saudou, F., Hen, R., 1994. 5-Hydroxytryptamine receptor subtypes in vertebrates and invertebrates. Neurochem. Int. 25, 503–532.

Stanton, J.A., Beer, M.S., 1997. Characterisation of a human 5-HT_{1A} receptor cell line using [^{35}S]GTPγS binding. Eur. J. Pharmacol. 320, 267–275.

Traynor, J.R., Nahorski, S.R., 1995. Modulation by μ-opioid agonists of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. Mol. Pharmacol. 47, 848–854.

Waeber, C., Grailhe, R., Yu, X.-J., Hen, R., Moskowitz, M.A., 1997. G-protein coupled 5-HT_5 receptors: localization in the CNS and lack of effect in the inhibition of dural protein extravasation. Advances in Serotonin Receptor Research (8–10 October 1997, San Francisco, USA) Abstr., AB33.

Wieland, T., Jakobs, K.H., 1994. Measurement of receptor-stimulated guanosine 5′-*O*-(γ-thio)triphosphate binding by G proteins. Methods Enzymol. 237, 3–13.

Wisden, W., Parker, E.M., Mahle, C.D., Grisel, D.A., Nowak, H.P., Yocca, F.D., Felder, C.C., Seeburg, P.H., Voigt, M.M., 1993. Cloning and characterization of the rat 5-HT_{5B} receptor. FEBS Lett. 333, 25–31.