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Functional coupling of a recombinant Human 5-HT_{5A} receptor to G-proteins in HEK-293 cells

[1,2]P.T. Hurley, [1,2]R.A. McMahon, [2]P. Fanning, [2]K.M. O'Boyle, [1,3]M. Rogers & [1,2,4]F. Martin

[1]National Agriculture and Veterinary Biotechnology Centre (NAVBC) and Departments of [2]Pharmacology and [3]Zoology, University College Dublin, Belfield, Dublin 4, Ireland

1 We have cloned, expressed and pharmacologically characterized the Human 5-HT_{5A} receptor.

2 We have shown that ligand activation of the Human 5-HT_{5A} receptor results in functional coupling to G-proteins in HEK-293 cells.

3 Stimulation of the receptor with 5-CT (5-carboxamidotryptamine) resulted in a dose-dependent increase in the % [^{35}S]-GTPγS binding over the basal level. This is the first study to describe such G-protein activation for the Human 5-HT_{5A} receptor in any cell.

4 A dose-dependent inhibition of cyclic AMP accumulation was observed in the recombinant Human 5-HT_{5A} receptor cell line, suggesting a functional coupling to a Gαi, G-protein in the HEK-293 cell line.

5 A ligand-stimulated reduction in the detectable level of the catalytic domain of protein kinase A (PKA) in nuclear extracts isolated from Human 5-HT_{5A} expressing cells was observed. This observation was consistent with the reduction in the level of cyclic AMP accumulation, in response to receptor activation.

Keywords: Human 5-HT_{5A}; G-protein coupled receptor; [^{35}S]-GTPγS; cyclic AMP

Introduction

The neurotransmitter 5-HT (5-hydroxytryptamine) mediates its physiological effects through a diverse set of receptors which regulate a variety of signal transduction pathways. With the help of molecular cloning techniques, fourteen receptor subtypes have been identified for 5-HT and these have been divided into 7 classes (5-HT_1–5-HT_7). The known receptors belong to 2 different protein superfamilies: (1) the G-protein coupled 7 transmembrane receptor superfamily or (2) ligand-gated ion channels (5-HT_3). Of the G-protein coupled members, at least five are coupled to the inhibition of adenylyl cyclase (5-$HT_{1A,B,D,E,F}$), three are linked to phosphoinositide hydrolysis (5-$HT_{2A,B,C}$) and three have been shown to stimulate adenylyl cyclase activity (5-HT_4, 5-HT_6 and 5-HT_7) (Hoyer *et al*., 1994; Gerhardt & van Heerikhuizen, 1997).

While amino acid sequence analysis (hydropathy plots) (Rees *et al*., 1994) of the human 5-HT_{5A} receptor suggests that it is a member of the G-protein coupled 7 transmembrane receptor superfamily, direct evidence of G-protein activation by this receptor has not been obtained.

Human 5-HT_{5A} receptor expression is restricted to the brain. RT-PCR analysis of different brain areas show expression of RNA encoding this receptor in thalamus, cerebellum, substantia nigra, hypothalamus and caudate nucleus (Rees *et al*., 1994; Ori *et al*., 1997). Chromosome localization studies have shown that the Human 5-HT_{5A} receptor gene is localized to human chromosome 7 (7q36) (Matthes *et al*., 1992). The human mutation for holoprosencephaly type III (Hatziioannou *et al*., 1991) which results in abnormal brain development, is localized to the same region as the Human 5-HT_{5A} receptor on chromosome 7, suggesting a potential role for the 5-HT_{5A} receptor in the development of this disorder. It has also been suggested that the Human 5-HT_{5A} receptor may play an important role in mediating the action of 5-HT in cerebellar function (Ori *et al*., 1997).

Studies on the mouse 5-HT_{5A} (Matthes *et al*., 1992; Plassat *et al*., 1992) and rat 5-HT_{5A} (Erlander *et al*., 1993) receptor have described difficulty in detecting a functional association of this receptor with any second messenger pathway. The reasons for this remain unclear.

The aim of the present study was to clone and express the Human 5-HT_{5A} receptor in HEK-293 cells, to establish its G-protein coupling and to examine possible coupling to second messenger pathways. This paper describes direct measurement of G-protein coupling to the Human 5-HT_{5A} receptor using a sensitive, [^{35}S]-guanosine-5-*O*-(8-thiotriphosphate) ([^{35}S]-GTPγS) binding assay. Stimulation of the Human 5-HT_{5A} receptor with 5-carboxamidotryptamine (5-CT) caused an inhibition of forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation. A modulation of cyclic AMP-stimulated PKA activity by receptor activation was also detected.

Methods

Cloning and expression of the full length Human 5-HT_{5A}cDNA

A 1071 base pair product (open reading frame) was amplified using PCR from a human brain 5′-STRETCH PLUS cDNA library (Clontech). The oligonucleotide primers used in the PCR reaction had the following sequences: sense strand, 5′-CCATCGATGGATTTACCAGTGAA-3′ and anti-sense strand, 5′-GGGGTACCCCTCAGTGTTGCCTAGA-3′. Inclusion of restriction sites (*ClaI*/*KpnI*) within these oligonucleotides allowed cloning of the amplified cDNA into pBluescript SK II$^+$ (Stratagene) to facilitate double stranded plasmid sequencing. The primer sequences were derived from sequences lodged in the GenBank/EMBL database (accession numbers: X81411 and X81412) (Rees *et al*., 1994). PCR was carried out using the high fidelity polymerase, *Pwo* (Boehringer Man-

[4] Author for correspondence.

nheim), for a total of 40 cycles under the following conditions: denaturation at 94° C for 45 s, annealing at 55° C for 45 s and extension at 72° C for 90 s. The product was cloned and double-stranded plasmid sequencing (Sequenase Version 2 Kit-USB) carried out on both strands to confirm the sequence of the cDNA. The confirmed Human 5-HT$_{5A}$ receptor cDNA was subcloned into the mammalian cell expression vector, pCI-neo (Promega).

Cell culture and cell membrane preparation

HEK-293 cells were maintained under standard culturing conditions for this cell line (ATCC: CRL 1573). Cells were transected with the pCI-neoh5-HT$_{5A}$ construct using the Ca^{2+} phosphate transfection procedure (Parker & Stark, 1979) and stable cell lines expressing the recombinant 5-HT$_{5A}$ receptor were established by G418 (geneticin) selection (1 mg ml^{-1}). The cell lines were subsequently grown in the presence of G418 (250 μg ml^{-1}). Cell membranes were prepared according to the protocol described in Werner *et al.* (1991), but with the cell membranes being re-suspended in 5-CT binding buffer (100 mM Tris- HCl, 1 mM EDTA, pH 7.7 with addition of 0.1% ascorbic acid) (Rees *et al.*, 1994).

Radioligand binding assays

For saturation binding analysis, 750 μl of membrane preparation (30 μg protein, as determined using the Bradford protein assay (Biorad)) was incubated with [^{3}H]-5-CT (specific activity, 51.3 Ci mmol^{-1}) (0.25–20 nM) and binding buffer using a final assay volume of 1 ml. Tubes were incubated at 37°C for 30 min (Rees *et al.*, 1994). Reactions were terminated by the addition of 5 ml of ice-cold binding buffer followed by rapid filtration through GF/B (Whatmann) filters using a Brandel cell harvester, followed by 2 washes with ice-cold binding buffer. Retained radioactivity was measured by liquid scintillation counting. Non-specific binding was defined by the inclusion of 10 μM methiothepin. Competition experiments were performed using 2 nM [^{3}H]-5-CT, and varying concentrations of the competing ligand. Non-specific binding was defined as described above. Data were analysed using the programme, GraphPad Inplot 4.

[^{35}S]-GTPγS binding assay

The protocol used was based on previous methods described for the [^{35}S]-GTPγS binding assay (Lazareno & Birdsall, 1993; Lorenzen *et al.*, 1993). Monolayer cultures were washed twice with phosphate buffered saline and the cells were lysed with 1 mM Tris-HCl, pH 7.5 for 15 min. Cell contents were collected by scraping and centrifuged at 40,000 *g* at 4°C for 15 min. Membrane pellets were re-suspended in TME buffer (75 mM Tris, 12.5 mM $MgCl_2$, 1.5 mM EDTA, pH 7.5) to give a final membrane protein concentration of 1 mg ml^{-1} (stored at −70°C). Membranes were homogenized using a Polytron before use. Pre-incubation with 10 μM GDP on ice for 10–20 min was carried out in order to suppress basal binding in each assay tube. Reactions were carried out in a 100 μl volume containing [^{35}S]-GTPγS (1,255 Ci mmol^{-1}) (50,000 c.p.m) (0.3–0.5 nM), drug diluted in incubation buffer (50 mM Tris-HCl, pH 7.4 containing 100 mM NaCl, 5 mM $MgCl_2$ 1 mM DTT, 1 mM Na_2 EDTA) and membrane homogenate; these were incubated at 30°C for 45 min. Non-specific binding was defined by the inclusion of 10 μM unlabelled GTPγS and was less than 1% of total binding. Reactions were again terminated by filtration through GF/C filters (Whatmann) as in radioligand binding assays. The filters were washed twice with ice-cold wash buffer (15 mM Tris-HCl, pH 7.4 containing 5 mM $MgCl_2$). Retained radioactivity was measured by liquid scintillation counting. Dose-response curves were constructed for 5-CT and from these, the % increase over basal and the concentrations required to produce half-maximal response (EC_{50}) were calculated using the GraphPad Inplot 4 programme.

Measurement of cyclic AMP accumulation

The method used was a modification of that described by Rugg and Simmons (1984). Cells grown to confluency in 24-well plates were washed with non-supplemented medium containing 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), pH 7.5 Cells were pre-incubated for 20 min in the presence of 0.5 mM isobutylmethylxanthine (IBMX). Forskolin (1 μM) and the appropriate concentration of 5-CT were added to pre-incubation medium and incubated with the cells at 37°C for 10 min. The reaction was terminated by aspiration of the medium, and the cells exposed to 0.2 M HCl for 10 min at 4°C. After neutralization with 0.2 M NaOH, extracts were centrifuged at 14,000 r.p.m. for 5 min at 4°C. The supernatant was assayed for cyclic AMP content using an assay based on that by Brown (1970). The assay was based on the competition of unlabelled cyclic AMP with a fixed amount of [^{3}H]-cyclic AMP (specific activity, 50 Ci mmol^{-1}) (9 nM) for binding to a protein with high affinity for cyclic AMP. Separation of protein-bound cyclic AMP from unbound cyclic AMP was achieved by filtration over GF/B filters as described for the radioligand binding assay. The amount of protein-bound labelled cyclic AMP detected was inversely proportional to the amount of unlabelled cyclic AMP in the sample.

Detection of protein kinase A activation

Cyclic AMP is capable of binding to the regulatory domain of PKA, causing release of the catalytic domain and allowing its translocation to the nucleus (Nigg *et al.*, 1985). Cells were incubated with forskolin in the presence/absence of 5-CT, as described for cyclic AMP measurements. Following this incubation, the cells were harvested by scraping and nuclear extracts prepared from each sample (Marti *et al.*, 1994). Nuclear extract (10 μg protein) from each treated sample was incubated for 30 min at 30°C with a fluorescent kemptide derivative according to the manufacturer's instructions (Promega). Phosphorylation of the peptide was visualised on a u.v. transilluminator after electrophoresis on a 0.8% agarose gel containing 50 mM Tris-HCl, pH 8.0.

Drugs and chemicals used

[^{3}H]-5-CT, [^{35}S]-GTPγS (NEN); [^{3}H]-cyclic AMP (Amersham); 5-CT and methiothepin (RBI); 5-HT, methysergide, (−)-propranolol, geneticin (G418), GDP, GTP, GTPγS, cyclic AMP and forskolin (Sigma). All other chemicals were obtained from local sources and were of high purity.

Results

Cloning, expression and characterization of the Human 5-HT$_{5A}$ receptor

The Human 5-HT$_{5A}$ receptor cDNA isolated was amplified as a 1071 bp open reading frame (encoding 357 amino acids)

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1/1       cca tcg atg gat tta cca gtg aac cta acc tcc ttt tcc ctc tcc acc ccc tcc cct ttg
           ClaI     M   D   L   P   V   N   L   T   S   F   S   L   S   T   P   S   P   L
61/21     gag acc aac cac agc ctc ggc aaa gac gac ctg cgc ccc agc tcg ccc ctg ctc tcg gtc
            E   T   N   H   S   L   G   K   D   D   L   R   P   S   S   P   L   L   S   V
121/41    ttc gga gtg ctt att ctc acc ttg ctg ggc ttt ctg gtg gcg gcg acg ttc gcc tgg aac
            F   G   V   L   I   L   T   L   L   G   F   L   V   A   A   T   F   A   W   N
181/61    ctg ctg gtg ctg gcg acc atc ctc cgt gta cgc acc ttc cac cgc gtg ccc cac aac ctg
            L   L   V   L   A   T   I   L   R   V   R   T   F   H   R   V   P   H   N   L
241/81    gtg gca tcc atg gcc gtc tcg gat gtc ctg gtg gcc gcg ctg gtc atg ccg ctg agc ctg
            V   A   S   M   A   V   S   D   V   L   V   A   A   L   V   M   P   L   S   L
301/101   gtg cac gag ctg tcc ggg cgc cgc tgg cag cta ggt cgg agg ctg tgc cag ctt tgg atc
            V   H   E   L   S   G   R   R   W   Q   L   G   R   R   L   C   Q   L   W   I
361/121   gcg tgc gac gtg ctt tgc tgc acg gcc agc atc tgg aac gtg acg gcc ata gcc ctg gac
            A   C   D   V   L   C   C   T   A   S   I   W   N   V   T   A   I   A   L   D
421/141   cgc tac tgg tcc atc acg cgc cac atg gaa tac acg ctc cgc acc cgc aag tgc gtc tcc
            R   Y   W   S   I   T   R   H   M   E   Y   T   L   R   T   R   K   C   V   S
481/161   aac gtc atg atc gcg ctc acc tgg gca ctc tcc gct gtc atc tct ctg gcc ccg ctg ctt
            N   V   M   I   A   L   T   W   A   L   S   A   V   I   S   L   A   P   L   L
541/181   ttt ggc tgg gga gag acg tac tct gag ggc agc gag gag tgc cag gta agc cgc gag cct
            F   G   W   G   E   T   Y   S   E   G   S   E   E   C   Q   V   S   R   E   P
601/201   tcc tat gcc gtg ttc tcc acc gta ggc gcc ttc tac ctg ccg ctc tgt gtg gtg ctc ttc
            S   Y   A   V   F   S   T   V   G   A   F   Y   L   P   L   C   V   V   L   F
661/221   gtg tac tgg aag atc tac aag gct gcc aag ttc cgc gtg ggc tcc agg aag acc aat agc
            V   Y   W   K   I   Y   K   A   A   K   F   R   V   G   S   R   K   T   N   S
721/241   gtc tca ccc ata tcc gaa gct gtg gag gtg aag gac tct gcc aaa cag ccc cag atg gtg
            V   S   P   I   S   E   A   V   E   V   K   D   S   A   K   Q   P   Q   M   V
781/261   ttc acg gtc cgc cac gcc acc gtc acc ttc cag cca gaa ggg gac acg tgg cgg gag cag
            F   T   V   R   H   A   T   V   T   F   Q   P   E   G   D   T   W   R   E   Q
841/281   aag gag cag cgg gcc gcc ctc atg gtg ggc atc ctc att ggc gtg ttc gtg ctc tgc tgg
            K   E   Q   R   A   A   L   M   V   G   I   L   I   G   V   F   V   L   C   W
901/301   atc ccc ttc ttt ctc acc gag ctc atc agt ccc ctc tgc tcc tgt gac atc ccc gcc atc
            I   P   F   F   L   T   E   L   I   S   P   L   C   S   C   D   I   P   A   I
961/321   tgg aaa agc atc ttc ctg tgg ctt ggc tac tcc aac tcc ttc ttt aac ccc ctg atc tat
            W   K   S   I   F   L   W   L   G   Y   S   N   S   F   F   N   P   L   I   Y
1021/341  acg gct ttc aac aag aac tac aac agc gcc ttc aag aac ttc ttt tct agg caa cac tga
            T   A   F   N   K   N   Y   N   S   A   F   K   N   F   F   S   R   Q   H   *
1081/361  ggg gta ccc c
             Kpn1
```

Figure 1 DNA and amino acid sequence of the human 5-HT$_{5A}$ receptor. Restriction sites are underlined.

(Figure 1). Sequencing showed the cDNA to be identical to that of exon 1 and 2 of the receptor previously lodged in the GenBank/EMBL database (Rees *et al*., 1994). The Human 5-HT$_{5A}$ receptor amino acid sequence is not closely related to that of any of the other known 5-HT receptors. The highest homology to other mammalian 5-HT receptors is approximately 38% to the 5-HT$_1$ family (Rees *et al*., 1994); however, comparisons with individual members of the 5-HT$_1$ family have not yet been reported. Figure 2 shows sequence alignment for the human 5-HT$_{1A}$, 5-HT$_{1F}$ and the 5-HT$_{5A}$ receptors. The Human 5-HT$_{1A}$ and 5-HT$_{1F}$ receptors have the highest sequence identity at the amino acid level among members of the Human 5-HT$_1$ family. These receptors share 37% and 38% identity, respectively, with the Human 5-HT$_{5A}$ receptor. However, although the 5-HT$_{5A}$ and 5-HT$_1$ peptide sequences seem related, certain structural features of the 5-HT$_{5A}$ receptor gene differ from that of the 5-HT$_1$ class, such as the presence of an intron between the sequences encoding transmembrane regions 5 and 6 (Rees *et al*., 1994). In contrast, the coding region for the 5-HT$_1$ receptors is intronless (Hoyer *et al*., 1994).

The cDNA was subcloned into the mammalian expression vector, pCI-neo and the resulting plasmid used to transfect HEK-293 cells. A number of cell lines stably expressing the receptor were obtained. Data analysis from the saturation binding studies carried out on a stably expressing recombinant 5-HT$_{5A}$ receptor HEK-293 cell line (Figure 3) yielded expression levels (B_{max}) of 2.1 ± 0.5 pmol receptor mg^{-1} membrane protein (mean $\pm$ s.e.mean, $n=3$) and a K_d value of 7.5 ± 2.5 nM (mean $\pm$ s.e.mean, $n=3$). Ligand binding studies for the receptor gave the pharmacological profile shown in Figure 4. The associated K_i values are tabulated in Table 1.

Detection of direct coupling of the recombinant Human 5-HT$_{5A}$ receptor to G-proteins

It is well established that the 5-HT$_{1B}$ receptor is negatively coupled to adenylyl cyclase through G-protein recruitment (Watson & Arkinstall, 1994) and hence HEK-293 membranes expressing recombinant rat 5-HT$_{1B}$ receptor (McMahon, 1995, unpublished) were used as a positive control in this study. Dose-dependent increases in binding of the non-hydrolyzable radiolabelled GTP analogue, [^{35}S]-GTPγS, in response to 5-CT were obtained for both 5-HT receptors tested (Figure 5). The rat 5-HT$_{1B}$ expressing membranes showed a maximal $26.2 \pm 3.1\%$ (mean $\pm$ s.e.mean, $n=5$) increase in [^{35}S]-GTPγS binding over basal levels. The Human 5-HT$_{5A}$ receptor gave a maximal $18.8 \pm 3.1\%$ (mean $\pm$ s.e.mean, $n=5$) stimulation over basal. The concentrations of 5-CT required to cause half-maximal stimulation (EC_{50}) of [^{35}S]-GTPγS binding by rat 5-HT$_{1B}$ and 5-HT$_{5A}$ receptors were 2.9×10^{-7} M and 8.5×10^{-7} M, respectively. Membranes prepared from untransfected HEK-293 cells showed no such dose-dependent increase in binding of the GTP analogue as compared to that obtained for membranes transfected with the 5-HT$_{5A}$ receptor (Figure 5).

Inhibition of forskolin-stimulated cyclic AMP accumulation by Human 5-HT$_{5A}$ receptor activation

The inhibition of forskolin-stimulated cyclic AMP accumulation by 5-CT was investigated in HEK-293 cells stably expressing the Human 5-HT$_{5A}$ receptor. The rat 5-HT$_{1B}$ expressing HEK-293 cell line acted as the positive control. In both these cell lines 5-CT inhibited forskolin-stimulated cyclic

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5-HT-1F : MD----FLNSSDQNLTSEELLN-------------RMPSKILVSLTLSGLALMTTTINSLVIAAI :  48
5-HT-1A : MD----VLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVAAI :  61
5-HT-5A : MDLPVNLTSFSLSTPSPLETNHSLGKDDLRPSSPLLSVFGVLILTLLGFLVAATFAWNLLVLATI :  65

5-HT-1F : IVTRKLHHPANYLICSLAVTDFLVAVLVMPFSIVYIVRE-SWIMGQVVCDIWLSVDITCCTCSIL : 112
5-HT-1A : ALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLN-KWTLGQVTCDLFIALDVLCCTSSIL : 125
5-HT-5A : LRVRTFHRVPHNLVASMAVSDVLVAALVMPLSLVHELSGRRWQLGRRLCQLWIACDVLCCTASIW : 130

5-HT-1F : HLSAIALDRYRAITDAVEYARKRTPKHAGIMITIVWIISVFISMPPL--FWRHQGTSRDDECIIK : 175
5-HT-1A : HLCAIALDRYWAITDPIDYVNKRTPRRAAALISLTWLIGFLISIPPMLGWRTPEDRSDPDACTIS : 190
5-HT-5A : NVTAIALDRYWSITRHMEYTLRTRKCVSNVMIALTWALSAVISLAPLLFGWGETYSEGSEECQVS : 195

5-HT-1F : HDHIVSTIYSTFGAFYIPLALILILYYKIYRAA-----------KTLYHKRQASRIA---KEEV : 225
5-HT-1A : KDH-GYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTGADTRHGASPAPQPKKSV : 254
5-HT-5A : REP-SYAVFSTVGAFYLPLCVVLFVYWKIYKAAKFRVG-------------------------- : 232

5-HT-1F : NGQV-----LLESGEKSTKSVSTSYVLEKSLSDPSTDFDKIHSTVRS-----LRSE--------- : 271
5-HT-1A : NGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVHRVGNSKEHLPLPSEAGPTPCAPA : 319
5-HT-5A : -----------SRKTNSVSPISEAVEVKDSAKQPQMVFTVRHATV------------------- : 266

5-HT-1F : -FKHEKSWR---RQKISGTRERKAATTLGLILGAFVICWLPFFVKELVVNVCD-KCKISEEMSNF : 331
5-HT-1A : SFERKNERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCESSCHMPTLLGAI : 384
5-HT-5A : TFQPEGDTWREQK-------EQRAALMVGILIGVFVLCWIPFFLTELISPLC--SCDIPAIWKSI : 322

5-HT-1F : LAWLGYLNSLINPLIYTIFNEDFKKAFQKLVRCRC--- : 366
5-HT-1A : INWLGYSNSLLNPVIYAYFNKDFQNAFKKIIKCKFCRQ : 422
5-HT-5A : FLWLGYSNSFFNPLIYTAFNKNYNSAFKNFFSRQH--- : 357
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Figure 2 Alignment of the human 5-HT$_{1A/F}$ and the human 5-HT$_{5A}$ amino acid sequence. Conserved amino acids are shaded.

AMP accumulation in a dose-dependent manner with EC_{50} values of 1.6×10^{-8} M for the 5-HT$_{5A}$ cell line and 1.8×10^{-9} M for the 5-HT$_{1B}$ cell line. The maximal inhibition of cyclic AMP accumulation obtained for the Human 5-HT$_{5A}$ receptor was $29.4 \pm 6.3\%$ (mean $\pm$ s.e.mean, $n = 5$), whereas activation of the rat 5-HT$_{1B}$ receptor led to a complete inhibition, reducing cyclic AMP accumulation to a level of $2.1 \pm 1.1\%$ of maximal (Figure 6). 5-CT had no effect on cyclic AMP accumulation in untransfected cells (data not shown).

Inhibition of activated nuclear PKA accumulation following ligand activation of the Human 5-HT$_{5A}$ receptor

The effect of Human 5-HT$_{5A}$ receptor stimulation on nuclear accumulation of the catalytic domain of PKA, in response to forskolin, was determined by visualizing phosphorylation of a fluorescent kemptide analogue (Marti *et al*., 1994) (Figure 7). Cells stimulated with 1 μM forskolin showed a large increase in kemptide phosphorylation as a result of cyclic AMP stimulated translocation of activated PKA to the nucleus, as judged by agarose gel electrophoresis. Following incubation of the 5-HT$_{5A}$ receptor expressing cells with 5-CT, a dose-dependent decrease in the amount of the phosphorylated kemptide was observed (Figure 7). Nuclear extracts isolated

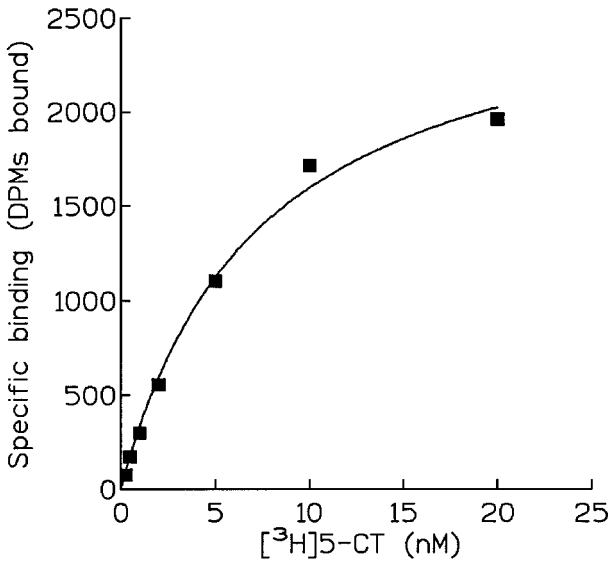

Figure 3 Saturation analysis of specific [^{3}H]-5-CT binding to HEK cell membranes expressing the human 5-HT$_{5A}$ receptor. Data points are mean duplicate values, and the curve is representative of 3 independent experiments.

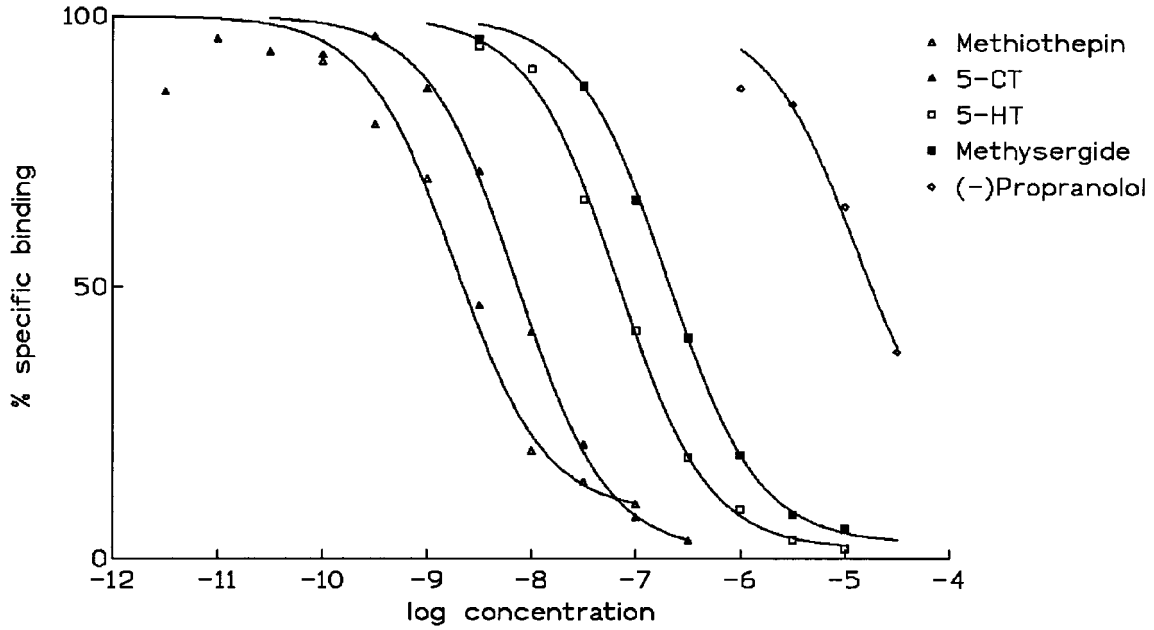

Figure 4 Competition for [^{3}H]-5-CT binding by increasing concentrations of the indicated ligands to 5-HT$_{5A}$ receptor expressing HEK-293 cell membranes. Values shown are mean duplicate values, and the curves are representative of 3 independent experiments.

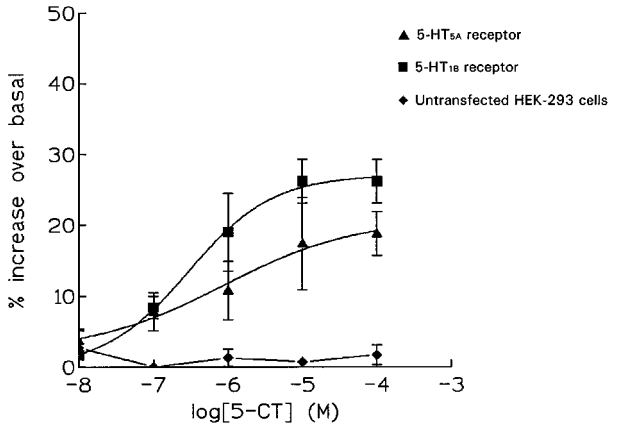

Figure 5 [^{35}S]-GTPγS binding upon agonist (5-CT) stimulation. Data are represented as the mean and vertical lines show s.e.mean ($n \geqslant 3$) for human 5-HT$_{5A}$ HEK-293 cell membranes, rat 5-HT$_{1B}$ HEK-293 cell membranes and untransfected HEK-293 cell membranes.

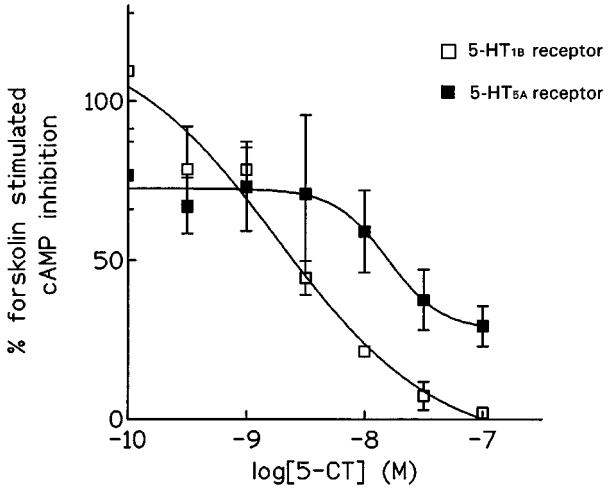

Figure 6 Inhibition of forskolin-stimulated cyclic AMP accumulation by 5-CT in stable transfectants expressing the indicated receptor; human 5-HT$_{5A}$ receptor or rat 5-HT$_{1B}$ receptor. Data are the mean and vertical lines show s.e.mean ($n \geqslant 3$).

Table 1 Affinity values (K_i) of various ligands for the cloned human 5-HT$_{5A}$ receptor

Compund	K_i (nM)
Methiothepin	1.6 ± 0.1
5-CT	4.6 ± 0.9
5-HT	54.4 ± 1.8
Methysergide	129 ± 29.0
(−)-Propranolol	9105 ± 1095.0

from rat 5-HT$_{1B}$ expressing cells also showed the expected reduction in phosphorylated substrate in response to increasing concentrations of 5-CT (Figure 7). No effects were obtained when untransfected HEK-293 were treated identically (data not shown).

Discussion

We have cloned a cDNA encoding a Human 5-HT$_{5A}$ receptor and characterized the functional properties of this receptor following its stable expression in HEK-293 cells. The pharmacological profile obtained was methiothepin $\geqslant$ 5-CT > 5-HT $\geqslant$ methysergide > (−)-propranolol. The affinity constants for these ligands are in agreement with those previously obtained for a recombinant Human 5-HT$_{5A}$ receptor when transiently expressed in CosM6 cells (Rees *et al*., 1994). The unusually high affinity of methiothepin for the receptor obtained in transiently transfected CosM6 cells was also seen in the stably transfected HEK-293 cells used in this study. This gives the Human 5-HT$_{5A}$ receptor a distinctly different pharmacology from that of other 5-HT receptors.

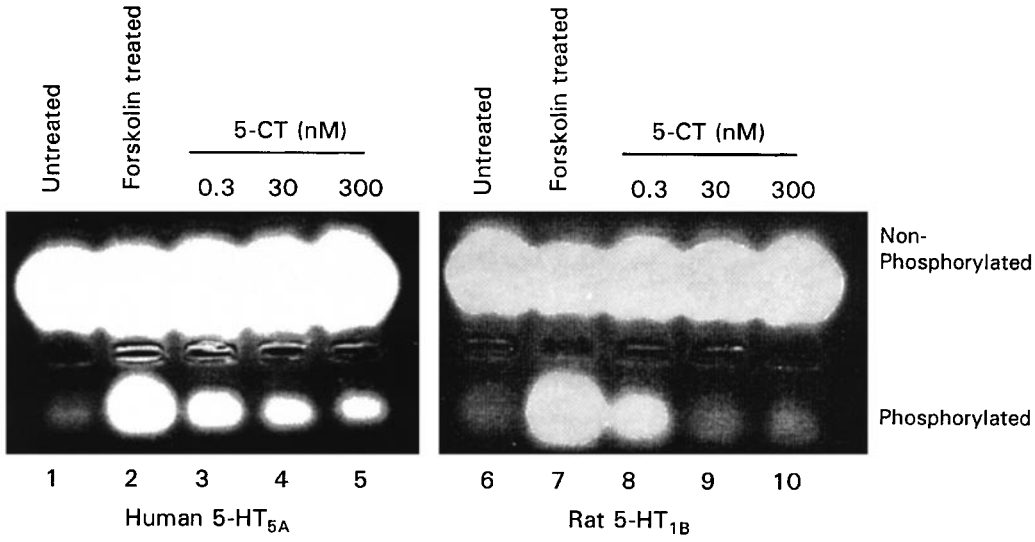

Figure 7 Protein kinase A (PKA) activity in nuclear extracts prepared from cells expressing the indicated receptor after treatment with 1μM forskolin in the presence/absence of 5-CT. Nuclear extracts from human 5-HT$_{5A}$ receptor expressing cells and nuclear extracts from rat 5-HT$_{1B}$ expressing cells were untreated or treated with 0.3–300 nM 5-CT. The phosphorylated peptide was separated from the non-phosphorylated peptide by agarose gel electrophoresis. Data are representative of 3 independent experiments.

The dose-dependent increase in GTPγS binding indicated a direct activation of the Human 5-HT$_{5A}$ receptor in response to ligand binding. The smaller response obtained for the 5-HT$_{5A}$ receptor (18.8% compared to 26.2% for the rat 5-HT$_{1B}$ receptor) may be due to inherent poor coupling of recombinant human 5-HT$_{5A}$ receptors. Previous studies carried out with recombinant Human 5-HT$_{5A}$ receptors (Matthes *et al*., 1992; Plassat *et al*., 1992; Erlander *et al*., 1993) have described difficulty in detecting activation of down-stream second messenger events, possibly due to inefficient association with G-proteins in the cell lines tested. However, there are 2 studies describing detectable down-stream events among the 5-HT$_5$ sub-family. Wisden *et al*. (1993) described guanosine nucleotide modulation of agonist binding to rat 5-HT$_{5B}$ receptors expressed in COS cells. In the presence of the non-hydrolyzable GTP analogue, Gpp(NH)p, the percentage of receptors in the high affinity state decreased from 25% to 9% suggesting that the receptor couples to G-proteins in COS cell membranes. Carson *et al*. (1996) suggested that the mouse 5-HT$_{5A}$ receptor is negatively coupled to adenylyl cyclase in astrocytes, as evident from the forskolin-stimulated reduction in cyclic AMP accumulation in response to 5-HT activation.

The trend in results obtained in the second messenger assays described here also suggest a weak coupling of the Human 5-HT$_{5A}$ receptor to G-proteins in HEK cells. Almost maximal inhibition of cyclic AMP accumulation was obtained with the 5-HT$_{1B}$ receptor, whereas the Human 5-HT$_{5A}$ receptor gave a reduction to only 29.4$\pm$6.4% of total accumulation (Figure 6) indicating a possible weaker/inefficient coupling to G-protein. The difference in EC$_{50}$ values obtained for the stimulation of [^{35}S]-GTPγS binding (e.g. 8.5×10^{-7} M for human 5-HT$_{5A}$ receptor) and for the inhibition of forskolin-stimulated cyclic AMP accumulation (e.g. 1.6×11^{10} M for the human 5-HT$_{5A}$ receptor) may reflect the degree of amplification (Ross, 1989) at the level of cyclic AMP accumulation.

Nigg *et al*. (1985) demonstrated nuclear accumulation of the catalytic domain of PKA in response to cyclic AMP treatment of MDBK cells. The nuclear PKA might specifically target transcription factors such as CREB (cyclic AMP response element binding protein) for phosphorylation and thereby influence transcription acutely. Inhibition of forskolin-stimulated nuclear accumulation of activated PKA in response to 5-CT occupation of the 5-HT$_{5A}$ receptor provided further evidence for G-protein coupling of this recombinant receptor and of its being linked to inhibition of adenylyl cyclase. Again, however, the dose-dependent reduction in forskolin-induced PKA nuclear translocation was less complete than was observed for the rat 5-HT$_{1B}$ receptor (Figure 7).

These results indicate that the Human 5-HT$_{5A}$ receptor can functionally couple to G-proteins. The particular subtype of G-protein subunit through which the receptor is mediating its effects remains unknown. It appears that the Human 5-HT$_{5A}$ receptor functions via a similar mechanism to the rat 5-HT$_{1B}$ receptor ($G_{\alpha i}/C_{\alpha o}$) (Watson & Arkinstall, 1994). However, the data suggest coupling to these G-proteins may be weak. The specific regulation of certain effector molecules such as adenylyl cyclase depends on the receptor subtype, receptor density and the environment (i.e. cell line) in which the receptor is operating (Sato *et al*., 1995). It may be the case that the particular G-protein subunit through which this receptor may function is in low abundance in the HEK cell line. In order to establish the specific G-protein through which the human 5-HT$_{5A}$ receptor may function, reconstitution experiments (Barr *et al*., 1997) could be used.

Reports on the expressed human dopamine D$_3$ receptor have also described difficulty in detecting efficient G-protein activation (Sibley & Monsma, 1992). It was suggested that it could not couple to G-proteins. However, recent studies show modulation of several second messenger systems, such as inhibition of cyclic AMP levels. Coupling to the pathway is weak compared to that shown for the D$_2$ dopamine receptor (Robinson *et al*., 1996). It thus seems likely that certain G-protein coupled receptor such as the Human 5-HT$_{5A}$ and D$_3$ receptors couple to G-proteins but only weakly when expressed in certain cell lines.

In conclusion, we show for the first time that ligand activation of Human 5-HT$_{5A}$ receptor results in functional coupling to G proteins in the HEK-293 cell line.

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