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Characterization of a human 5-hydroxytryptamine$_3$ receptor type A (h5-HT$_3$R-A$_S$) subunit stably expressed in HEK 293 cells

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1 A cloned cDNA encoding a human 5-hydroxytryptamine$_3$ receptor type A subunit (h5-HT$_3$R-A$_S$) was transfected into human embryonic kidney (HEK 293) cells maintained in cell culture and a stable cell line expressing a high density of the recombinant receptor was selected.

2 Membrane homogenates prepared from transfected, but not untransfected, cells exhibited a homogeneous and saturable population ($B_{max}=4.49\pm0.46$ pmol mg^{-1} protein) of sites that bound the radiolabelled 5-HT$_3$ receptor antagonist, [^{3}H]-granisetron with high affinity (p$K_D=8.87\pm0.08$). Kinetic studies (at 37°C) revealed rapid association ($k_{+1}=4.76\pm0.3\times10^8$ M^{-1} min^{-1}) and dissociation ($k_{-1}=0.21\pm0.003$ min^{-1}) of the radioligand.

3 Selective and non-selective 5-HT$_3$ receptor ligands competed for [^{3}H]-granisetron binding with a rank order of potency (granisetron > ondansetron > *meta*-chlorophenylbiguanide > 5-HT > 2-methyl-5-HT > metoclopramide >> phenylbiguanide > cocaine > (+)-tubocurarine) identical to that established for 5-HT$_3$ receptors endogenous to the human CNS.

4 In electrophysiological recordings performed on transfected cells, voltage-clamped at a holding potential of −60 mV, locally applied 5-HT (10 μM) evoked transient inward current responses that reversed in sign at a potential of -1.0 ± 1.1 mV. Such responses were antagonized in a reversible manner by granisetron (1 nM).

5 The construction of a stable cell line expressing a high density of recombinant human 5-HT$_3$ receptors which display appropriate pharmacology and function will assist in the further characterization of this receptor subtype and the exploration of species differences in 5-HT$_3$ receptor pharmacology.

Keywords: 5-Hydroxytryptamine$_3$ type A receptor subunit; 5-hydroxytryptamine; HEK 293 cells; [^{3}H]-granisetron; (+)-tubocurarine

Introduction

The 5-hydroxytryptamine type 3 (5-HT$_3$) receptor is a member of the family of ligand-gated ion channels that includes the nicotinic, GABA$_A$ and glycine receptor classes (Boess & Martin, 1994; Hoyer *et al.*, 1994; Jackson & Yakel, 1995; Lambert *et al.*, 1995). The receptor occurs upon elements of both the peripheral and central nervous systems, mediating fast excitatory responses to 5-HT via the gating of a cation-selective pore (Derkach *et al.*, 1989). During the development of the first generation of 5-HT$_3$ receptor-selective ligands, striking differences in antagonist affinities were encountered between 5-HT$_3$ receptor-containing preparations (Fozard, 1983; 1984; Richardson *et al.*, 1985). Although this was originally thought to reflect the existence of multiple 5-HT$_3$ receptor subtypes with tissue-dependent expression (Richardson & Engel, 1986), subsequent studies have identified inter-species differences in 5-HT$_3$ receptor pharmacology as the principle determinant of such heterogeneity (reviewed by Peters *et al.*, 1994). For example, the 5-HT$_3$ receptor expressed by guinea-pig tissues displays atypically low affinity towards 5-HT$_3$ receptor selective ligands and appears refractory to activation by the arylbiguanide class of agonist (Butler *et al.*, 1990; Newberry *et al.*, 1991; Wong *et al.*, 1993b; 1995). Additionally, both functional and radioligand binding studies indicate certain compounds (e.g. *m*-chlorophenylbiguanide; (+)-tubocurarine) able to discriminate between 5-HT$_3$ receptors present in mouse, rat and rabbit tissues (Newberry *et al.*, 1991; Peters *et al.*, 1991; Kilpatrick *et al.*, 1991; Wong *et al.*, 1993b). Radioligand binding assays characterizing human 5-HT$_3$ recognition sites in post-mortem brain tissue have revealed a pharmacology that is unlike that established for routinely studied laboratory animals such as the rat (Abi-Dargham *et al.*, 1993; Bufton *et al.*, 1993). By contrast to the literature documenting inter-species variants of 5-HT$_3$ receptor, there is only limited evidence to support the existence of intra-species subtypes (Bonhaus *et al.*, 1993; Wong *et al.*, 1993a; Hussy *et al.*, 1994; but see Perren *et al.*, 1995).

The relatively low density of 5-HT$_3$ receptors in human brain has impeded their characterization. Although the development of extremely high affinity radioligands has improved the sensitivity of binding assays (Wong *et al.*, 1995), the limitations associated with the use of post-mortem tissues remain. A solitary report (Maura *et al.*, 1992) describing 5-HT$_3$ receptor-mediated suppression of acetylcholine release from human cortical synaptosomes prepared from tissue from patients undergoing neurosurgery illustrates an alternative approach. However, for routine study, a continuous cell line expressing the human receptor would be of considerable value.

The cloning of a human 5-HT$_3$ receptor subunit from hippocampal and amygdala cDNA libraries by homology screening and its expression in *Xenopus laevis* oocytes and COS-1 cells have been recently reported (Belelli *et al.*, 1995; Hope *et al.*, 1995; Miyake *et al.*, 1995). The human subunit (termed h5-HT$_3$R-A$_S$), is a species homologue of an alternatively spliced form (Hope *et al.*, 1993) of the mouse subunit (5-HT$_3$R-A) originally isolated from the NCB-20 cell line by Maricq *et al.* (1991). A rat 5-HT$_3$R-A subunit has also been cloned and heterologously expressed (Johnson & Heinemann, 1992; Isenberg *et al.*, 1993; Miyake *et al.*, 1995). The recombinant receptors function efficiently as homo-oligomeric

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complexes and at least in the case of the thoroughly studied mouse homologue and its alternatively spliced form (Hope *et al.*, 1993), species-dependent pharmacology is preserved to a remarkable extent (Maricq *et al.*, 1991; Downie *et al.*, 1994; 1995; Gill *et al.*, 1995). To date, the 5-HT$_3$R-A subunit is the only structural component of the 5-HT$_3$ receptor that has been identified, fuelling speculation that endogenous receptors might occur as homo-pentameric complexes (reviewed by Jackson & Yakel, 1995).

In the present study, we describe the construction of a stably transfected cell line expressing the h5-HT$_3$R-A$_S$ at high density. The recombinant receptors display a pharmacological profile, closely resembling that established for 5-HT$_3$ receptors endogenous to human brain tissue and exhibit appropriate functional properties in electrophysiological assays. A preliminary account has appeared in abstract form (Hope *et al.*, 1995).

Methods

Cell culture and stable transfection of HEK 293 cells

The human embryonic kidney cell line HEK 293 (Harrison *et al.*, 1977), was maintained in minimal essential medium supplemented with 10% (v/v) foetal calf serum, streptomycin (100 mg l^{-1}) and penicillin (1×10^5 iu l^{-1}). The cells were seeded at a density of 2×10^5 cells per 75 cm^2 flask and maintained at 37°C in an atmosphere of 95% air/5% CO_2 at 100% relative humidity.

The h5-HT$_3$R-A$_S$ cDNA was cloned into the unique *EcoR1* restriction site in the eukaryotic expression vector pCNOD, under the control of the human cytomegalovirus promoter. For stable transfection, the recombinant DNA was linearized with *Pvu I* and introduced into HEK 293 cells using the lipofection reagent DOTAP (Gibco-BRL). The recombinant 5-HT$_3$R-A$_S$ cDNA (5 μg) and DOTAP (70 μl) were individually diluted to 250 μl in a HEPES buffered saline (HEPES 20 mM; NaCl 150 mM, pH 7.4). The two solutions were subsequently combined in 10 ml of growth medium and incubated with the HEK 293 cells at 37°C for 4 h. The cells were washed in a phosphate buffered saline (PBS) comprising (in mM): NaCl 140, KCl 2.7, Na_2HPO_4 9.6, KH_2PO_4 1.5, (pH 7.4) and incubated in normal culture medium for 48 h to allow for phenotypic expression of the h5-HT$_3$R-A$_S$ subunit. Stable transformants were selected in medium containing geneticin (0.75 mg ml^{-1}). Single colonies of geneticin resistant cells were isolated individually using cloning rings, transferred to separate wells in a 24 well culture dish and grown to confluence. Transformed cells were subsequently maintained in 75 cm^2 flasks in growth medium supplemented with geneticin.

RNA isolation and Northern blot analysis of transfected HEK 293 cells

HEK 293 cells stably expressing the h5-HT$_3$R-A$_S$ subunit were grown in 80 cm^2 flasks containing growth medium supplemented with geneticin, as described above. When confluent, the growth medium was removed and the cells were washed twice in 5 ml PBS. Total RNA was isolated essentially as described by Chirgwin *et al.* (1979). The cells were lysed in 3.5 ml of guanidinium thiocyanate homogenization buffer (guanidinium isothiocyanate 4 M, Tris-HCl, 0.1 M, 1% (v/v) β-mercaptoethanol, 0.5% (w/v) sodium lauryl sarcosinate; pH 7.4) and total RNA was isolated by centrifugation through a caesium chloride cushion (CsCl, 5.7 M, EDTA, 0.01 M, pH 8.0) at 103 000 *g* for 15 h at 20°C (Sambrook *et al.*, 1989).

Total RNA (5 μg) was resolved on a 1% denaturing agarose/7.4% formaldehyde gel, transferred to a Hybond N$^+$ (Amersham International) nylon membrane, in 20 × sodium chloride/sodium citrate solution (SSC) comprising: NaCl 3M, trisodium citrate 0.3 M (pH 7.0) and the filters were fixed by baking at 80°C for 2 h. Full length cDNA probes were radiolabelled by random priming with α^{32}P dCTP (Feinberg & Vogelstein, 1983) and hybridized with the Northern blotted RNA in 5 × SSPE, 12.5 M formamide, 5 × Denhardt's reagent, 0.5% (w/v) sodium dodecyl sulphate (SDS), 100 μg/ml^{-1} denatured herring sperm DNA, for 12–16 h at 42°C. Post-hybridization washes were performed twice in 0.1 × SSC, 0.1% SDS at 65°C for 15 min, prior to autoradiography against Hyperfilm MP X-ray film (Amersham International) at −70°C. The filters were stripped between hybridizations by incubating twice in boiling 0.5% SDS.

Radioligand binding

Membrane preparation HEK293 cells stably transfected with the 5-HT$_3$R-A$_S$ cDNA (passages 7–12 inclusive) were grown to confluence in 175 cm^2 flasks as described above. Following aspiration of the culture medium, cells were harvested by mechanical agitation in ice cold PBS, centrifuged at 4000 *g* for 10 min and subsequently stored as a cell pellet at −80°C. When required, the pellet was thawed and resuspended in ice cold homogenization buffer (Tris 50 mM, EGTA 5.0 mM, phenylmethylsulphonylfluoride 0.1 mM, pH 7.6) and homogenized in an Ultra Turrax tissue homogenizer (full speed for 10 s). The homogenate was centrifuged at 48 000 *g* for 10 min at 4°C. The resulting pellet was resuspended in ice cold binding buffer comprising (in mM): NaCl 140, KCl 2.8, $CaCl_2$ 1.0; $MgCl_2$, 2.0; HEPES 10 (pH 7.4) and centrifuged as above. The pellet was resuspended in ice cold binding buffer and the protein concentration was determined by the method of Lowry *et al.* (1953). The membrane homogenate was stored as aliquots at −70°C until required in radioligand binding assays (within 4 weeks).

Binding assay In saturation and competition studies the membrane homogenate was adjusted to a protein concentration of approximately 600 μg ml^{-1} in binding buffer (see above). Polypropylene assay tubes (in triplicate) contained 100 μl [^{3}H]-granisetron, 100 μl competing drug or buffer and 50 μl of membrane homogenate in a total reaction volume of 1 ml. Tubes were pre-warmed to 37°C and binding was initiated by the addition of the membrane homogenate and allowed to proceed for 60 min. Bound and free radioligand were separated by the addition of 3 ml of ice-cold buffer and immediate vacuum filtration through pre-soaked (0.1% (v/v) polyethyleneimine) Whatman GF/B filters mounted on a 'Millipore' filtration manifold. Filters were washed with a further 2 × 3 ml applications of buffer. In saturation studies, nine concentrations (0.1–10 nM) of [^{3}H]-granisetron were employed. In competition experiments, IC_{50} values were determined using 8 to 13 concentrations of the competing ligand.

In kinetic studies, binding assays were performed in 50 ml glass conical flasks, containing 1 nM [^{3}H]-granisetron, 0.95 mg membrane homogenate adjusted to a final volume of 30 ml with buffer. Binding was initiated by the addition of radioligand (3 ml) to the pre-warmed sample and allowed to proceed at 37°C. To determine the kinetics of association, 1 ml aliquots of the homogenate were withdrawn at specified time points and immediately filtered. Filters were rinsed with a further 3 × 3 ml applications of ice-cold buffer. In studies examining the dissociation of [^{3}H]-granisetron, binding was allowed to proceed to steady-state over a period of 60 min and net dissociation was initiated by the addition of a saturating concentration of ondansetron (300 μl; 10 μM final concentration); 1 ml samples were withdrawn at specific time points and processed as described above. In all assays, non-specific binding was defined by the presence of 10 μM ondansetron. Filter retained radioactivity was quantified by placing air-dried filters in 5 ml Optiphase Hi-safe 2 and conventional liquid scintillation spectroscopy at an efficiency of approximately 50%.

Electrophysiological recordings

Membrane currents evoked by 5-HT were recorded from transfected HEK 293 cells (passages 15–40) by the whole-cell recording mode of the patch-clamp technique as described by Gill *et al.* (1995). In brief, solitary HEK 293 cells were voltage clamped at −60 mV, unless stated otherwise, utilizing a List Electronics EPC-7 amplifier and converter headstage. Cells were continuously superfused (3–5 ml min^{-1}) with a solution containing (in mM): NaCl 140, KCl 2.8, $CaCl_2$ 1.0, $MgCl_2$ 2.0 and HEPES 10 (pH 7.2). The patch pipette solution which dialyzed the cell interior comprised (in mM): CsCl 140, $MgCl_2$ 2.0, $CaCl_2$ 0.1, EGTA 1.1 and HEPES 10 (pH 7.2).

Macroscopic current responses to 5-HT (10 μM) were evoked at a frequency of 0.05–0.02 Hz by brief (30–80 ms) applications of the agonist under pressure (1.4×10^5 Pa) from a second patch pipette. Antagonist compounds were applied via the superfusate for a time sufficient to ensure that blockade developed to a steady-state. Data were stored on a Racal store 4DS F/M tape recorder (500 Hz low-pass filtering) and also acquired digitally on line using an analogue to digital converter (sampling rate 100 Hz) and the WCP software package (Dempster, 1993) running on an IBM compatable PC. Electrophysiological experiments were conducted at ambient temperature (20–22°C).

Data analysis

Saturation, kinetic and competition data were analysed by computer assisted iterative curve fitting (Fig P version 6c; Biosoft, Cambridge). The maximal binding of [^{3}H]-granisetron at equilibrium (B_{max}) and the equilibrium dissociation constant (K_D) were derived directly from saturation curves fitted with a one site ligand binding model. Competition data were analysed according to the equation:

$$\frac{B_c}{B} = \frac{[C]^{n_H}}{[C]^{n_H} + [IC_{50}]^{n_H}} \quad (1)$$

where B and B_c are the binding observed in the absence and presence of the competing ligand C, IC_{50} is the molar concentration of C reducing specific binding by 50% and n_H is the pseudo Hill coefficient. The apparent dissociation constant (K_i) of the competing ligand was estimated from the Cheng-Prusoff equation (Cheng & Prusoff, 1973):

$$K_i = \frac{[IC_{50}]}{1 + [L]/K_D} \quad (2)$$

where L and K_D are the concentration and equilibrium dissociation constant of the radioligand respectively and IC_{50} is as previously defined. Association and dissociation rate constants were derived from fitting the first-order equations:

$$B_t = B_e(1 - \exp - (k_{obs}t)) \quad (3)$$

and

$$B_t = B_o \exp - (k_{-1}t) \quad (4)$$

respectively, where B_t is specific binding at time t, B_e is the fitted binding at equilibrium, k_{obs} is an observed pseudo first-order rate constant, B_O is equilibrium binding at $t=0$ prior to dissociation and k_{-1} is the dissociation rate constant. The association rate constant, k_{+1}, was calculated as:

$$k_{+1} = (k_{obs} - k_{-1})/[L] \quad (5)$$

Equations equivalent to 3 and 4 above were employed to derive the apparent K_D for granisetron from the kinetics of antagonism in electrophysiological studies. Data are reported as the mean ± standard error of the mean.

Drugs used

[^{3}H]-granisetron (3.1 TBq $mmol^{-1}$) was supplied by NEN and used as received. Drugs were obtained from the following sources: atropine sulphate, cocaine hydrochloride, cyproheptadine hydrochloride, dopamine hydrochloride, 8-OH-DPAT hydrobromide ((±)-8-hydroxy-2-(di-n-propylamino)-tetralin), 5-HT creatine sulphate, 5-methoxytryptamine hydrochloride, metoclopramide hydrochloride, (±)-noradrenaline hydrochloride, phentolamine hydrochloride, (−)-propranolol hydrochloride, (+)-tubocurarine chloride and yohimbine hydrochloride (Sigma Chemical Co, Poole, U.K.); *meta*-chlorophenylbiguanide (*m*-CPBG) dihydrochloride and ondansetron hydrochloride (Glaxo, Greenford, U.K.); granisetron hydrochloride (SmithKline Beecham, Harlow, U.K.); ketanserin tartrate (Janssen, Beerse, Belgium); 2-methyl-5-HT maleate and 1-phenylbiguanide (PBG) (Research Biochemicals Inc., Natick, MA, U.S.A.) and methysergide hydrogen maleate (Sandoz, Basel, Switzerland). Drugs were freshly prepared as concentrates in either buffer or twice distilled de-ionized water.

Results

Northern blot analysis of stably transfected cells

Expression of the h5-HT_3R-A_S subunit was initially assessed by Northern blot analysis employing an α^{32}P-labelled h5-HT_3R-A_S full length cDNA probe. Total RNA was isolated from 8 cell lines derived from distinct geneticin resistant colonies, as well as from control untransfected cells. The h5-HT_3R-A_S probe recognised a single mRNA species in 7 of the cell lines tested (Figure 1a), although the level of expression of mRNA in the individual cell lines varied markedly. No hybridization to RNA isolated from the untransfected HEK293 cells was detected. To confirm equal loading of each RNA sample, the Northern blot was stripped and re-probed with an α^{32}P-labelled actin full length cDNA (Leader *et al.*, 1985) which under the same conditions of stringency, recognized a single but distinct mRNA species with approximately the same intensity, in both transfected and non-transfected cells (Figure 1b). The cell line expressing the highest level of h5-HT_3R-A_S mRNA was selected for further analysis in radioligand binding assays.

Radioligand binding assays

Saturation and kinetics of [^{3}H]-granisetron binding The expression of the h5-HT_3R-A_S subunit in transfected HEK 293 cells was established in radioligand binding assays employing the selective high affinity antagonist [^{3}H]-granisetron (Nelson & Thomas 1989), a ligand previously employed to characterize human 5-HT_3 receptors in post-mortem human tissues (Bufton *et al.*, 1993). On membranes prepared from HEK 293 cells stably transfected with the cDNA encoding the h5-HT_3R-A_S subunit, [^{3}H]-granisetron demonstrated specific binding of high affinity to an apparently homogeneous and saturable population of binding sites ($pK_D = 8.87 \pm 0.08$; $B_{max} = 4.49 \pm 0.46$ pmol mg^{-1} prot; Hill coefficient $= 1.00 \pm 0.07$; $n = 3$) (Figure 2a). Non-specific binding (defined by the inclusion of ondansetron (10 μM)) increased linearly with radioligand concentration. As a consequence of the high level of expression achieved, the ratio of specific to non-specific binding was exceptionally favourable, the latter routinely accounting for approximately 4% of total binding at K_D. Only non-specific binding was detected in membranes prepared from untransfected HEK 293 cells (data not shown).

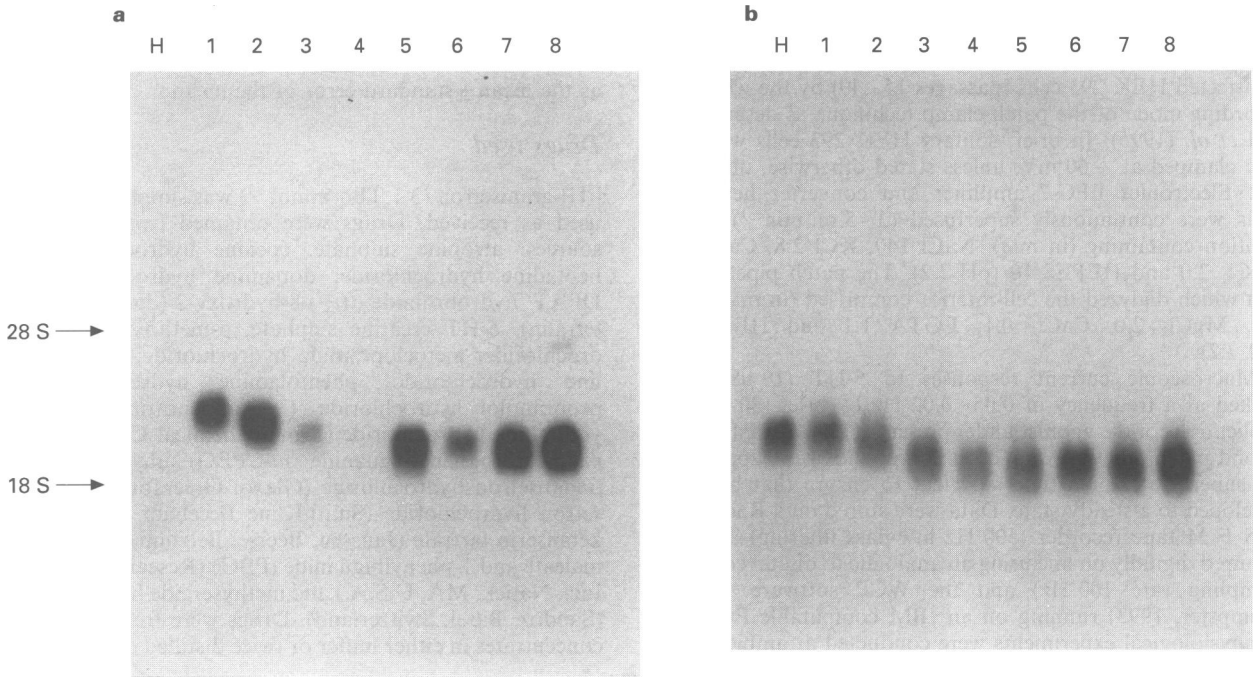

Figure 1 Northern blot analysis of HEK293 cells stably transfected with the h5-HT$_3$R-A$_S$ subunit. Total RNA was isolated from control, untransfected HEK293 cells (lane H), as well as from eight individual cell lines stably transfected with the h5-HT$_3$R-A$_S$ cDNA (lanes 1–8); 5 μg of total RNA isolated from each cell line was resolved on a 1% agarose/formaldehyde gel and transferred onto a Hybond N$^+$ nylon membrane. The hybridization was performed as described in the methods. (a) The filter was hybridized with the h5-HT$_3$R-A$_S$ cDNA radiolabelled with α [^{32}P]-dCTP and washed twice to high stringency in 0.1 × SSC, 0.1% SDS at 65°C for 15 min. (b) The filter was stripped and re-hybridized with an α [^{32}P] labelled γ actin pseudogene and washed as in (a). The position of the 28S and 18S ribosomal bands are indicated.

The binding of [^{3}H]-granisetron (1 nM) to membranes derived from transfected cells was rapid and completely reversible. The association and dissociation reactions were both monophasic (Figure 2b). Binding reached steady-state within 5 min and did not change appreciably thereafter. The observed association rate (k_{obs}) was 0.69 ± 0.03 min^{-1} ($n=3$). The dissociation of [^{3}H]-granisetron, elicited by the addition of 10 μM ondansetron, yielded a rate constant (k_{-1}) of 0.21 ± 0.003 min^{-1} ($n=3$). From the latter value and k_{obs}, the association rate constant (k_{+1}) was calculated to be $4.76 \pm 0.3 \times 10^8$ M^{-1} min^{-1} ($n=3$). The kinetically determined K_D (k_{-1}/k_{+1}) was 0.45 ± 0.03 nM ($n=3$), a value in reasonable agreement with that found in saturation experiments (i.e. 1.4 nM).

Competition studies Established agonists and antagonists of 5-HT$_3$ receptors competed for tritiated [^{3}H]-granisetron binding in a concentration-dependent and monophasic manner. Figure 3 illustrates representative data obtained with granisetron, ondansetron, (+)-tubocurarine (see discussion), *m*-CPBG and 5-HT. The pK_i values derived from the Cheng-Prusoff relationship of the compounds tested are listed in Table 1. At saturating concentrations, all agents documented as 5-HT$_3$ receptor ligands competed for a similar fraction of total [^{3}H]-granisetron binding ($\cong$96%), that remaining corresponding to non-specific binding defined by ondansetron (10 μM). In the case of antagonist compounds, pseudo Hill coefficients derived from the analysis of displacement curves were close to unity (range 1.07–1.25; Table 1). Slightly higher values (range 1.26–1.34) were encountered in the case of agonists (Table 1). Compounds demonstrating high affinity for various subtypes of G-protein-linked 5-HT receptor (e.g. methysergide, ketanserin) had no effect upon the binding of [^{3}H]-granisetron when tested at 10 μM. At the same concentration, agents interacting with adreno-, dopamine and muscarinic receptor subtypes competed either weakly for [^{3}H]-granisetron binding, or not at all (Table 1).

Electrophysiological recordings

At a holding potential of −60 mV, locally applied 5-HT (10 μM) elicited a transient inward current response from all transfected HEK 293 cells challenged with the agonist ($n=103$). 5-HT had no effect upon untransfected cells ($n=20$). Under such conditions, the peak current response recorded from transfected cells amounted to 984 ± 202 pA ($n=20$). The amplitude of the response decreased with membrane depolarization and reversed in sign at a potential of -1.0 ± 1.1 mV ($n=9$), consistent with the activation of a channel which conducts monovalent cations non-selectively. Granisetron, bath applied at a concentration (i.e. 1 nM) corresponding approximately to the K_D found in the saturation assays, exerted a slowly developing blockade of the current response to 5-HT (Figure 4a,b). Maximal depression of the response amplitude (to 6.9 ± 1.2% of control, $n=4$) was observed within 12–14 min of the introduction of antagonist-containing superfusate. Thereafter, superfusion with drug-free solution produced a progressive reversal of the blockade, but in most instances, recordings were lost before substantial recovery could be demonstrated. Under favourable conditions, where washout was followed for a period of approximately 1 h, more complete recovery was observed. In such instances, the time course of both the onset and reversal of blockade approximated to single exponential functions, allowing an estimation of the rate constants associated with these processes (k_{obs} and k_{-1} respectively) to be made. For the data illustrated in Figure 4a and b, k_{obs} and k_{-1} were 0.32 min^{-1} and 0.023 min^{-1} respectively, yielding a kinetic estimate of the apparent K_D for granisetron of 109 pM. A similar analysis conducted upon a second cell provided an apparent K_D of 192 pM. To ensure that the slow kinetics observed for granisetron were not an artifact of the method of drug application, blockade by cocaine was additionally examined in the above cells. As anticipated from its low affinity in the binding studies, antagonism by cocaine (3 μM, approximate K_i in competition studies) was quick to develop and reversed rapidly and completely (Figure 4c,d).

Discussion

A substantial body of evidence derived from functional and radioligand binding assays points to inter-species variation in the pharmacological properties of the 5-HT$_3$ receptor subtype (e.g. Peters *et al.*, 1992; 1994). A variety of models exist which permit detailed pharmacological characterization of 5-HT$_3$ receptors of laboratory animals (e.g. Sanger & Jones, 1994), but equivalent studies of the human 5-HT$_3$ receptor complex have relied largely upon the use of membrane homogenates prepared from post-mortem brain tissue. The density of 5-HT$_3$ receptors within human forebrain structures (e.g. hippocampus, amygdala, putamen and caudate) is modest (~7–18 fmol mg^{-1} protein; Bufton *et al.*, 1993; Abi-Dargham *et al.*, 1993; Wong *et al.*, 1995) and their detection has necessitated the use of a restricted number of selective high-affinity antagonists such as [^{3}H]-zacopride (Barnes *et al.*, 1989), [^{3}H]-granisetron (Bufton *et al.*, 1993), [^{3}H]-LY278584 (Abi-Dargham *et al.*, 1993) and, most recently, [^{3}H]-RS 25259-197 (Wong *et al.*, 1995). The aim of the present study was to provide an alternative system, suitable for both radioligand binding and functional assays, by stably expressing the recently cloned h5-HT$_3$R-A$_S$ subunit (Belelli *et al.*, 1995; Miyake *et al.*, 1995) in a human somatic cell line.

Membrane homogenates prepared from HEK 293 cells transfected with the h5-HT$_3$R-A$_S$ expressed a high density (≈4.4 pmol mg^{-1} protein) of [^{3}H]-granisetron-labelled recombinant receptors which displayed appropriate pharmacology and were functional in electrophysiological assays. The level of expression was approximately 10 fold higher than observed with [^{3}H]-YM060 binding to membranes prepared from COS-1 cells transiently transfected with the h5-HT$_3$R-A$_S$ cDNA (Miyake *et al.*, 1995). The binding of [^{3}H]-granisetron to membranes prepared from transfected HEK 293 cells was of a slightly higher affinity (K_D 1.4 nM in both saturation and competition studies) than that found in homogenates of human putamen (K_D 6.3 nM; Bufton *et al.*, 1993), but agreed with the value derived by Miyake *et al.* (1995) from competition studies with granisetron and [^{3}H]-YM060 conducted upon transiently transfected COS-1 cells. In the present study, [^{3}H]-granisetron exhibited apparently monophasic association and dissociation kinetics. The kinetics of [^{3}H]-granisetron binding

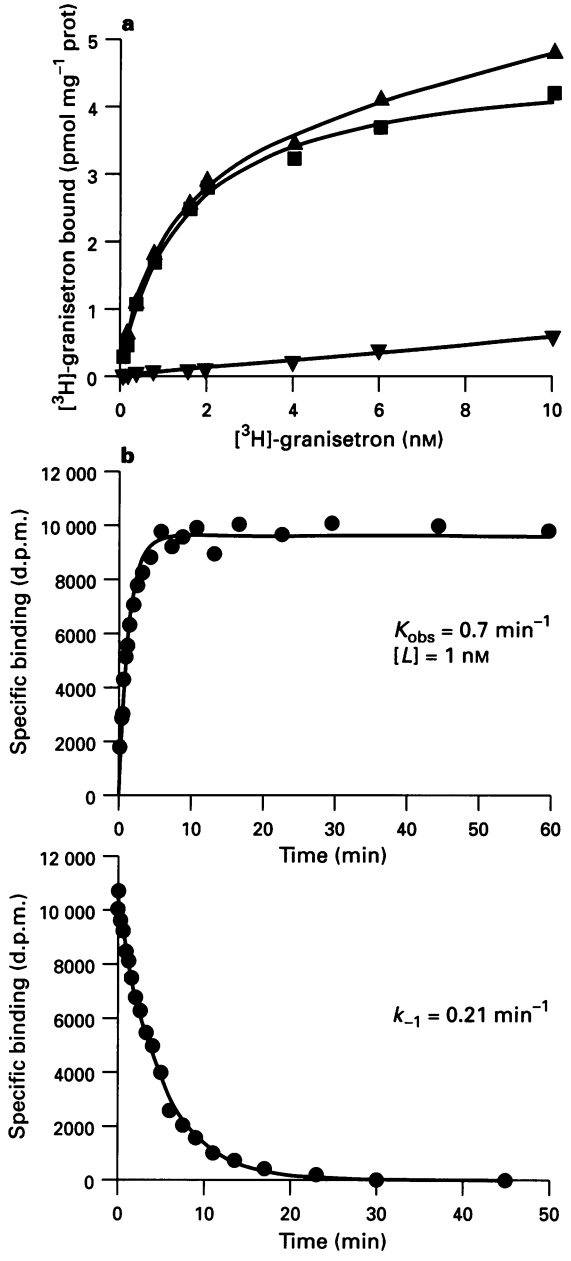

Figure 2 Characterization of [^{3}H]-granisetron binding to membrane homogenates prepared from HEK293 cells stably expressing the h5-HT$_3$R-A$_S$ cDNA. (a) Saturation studies: membrane homogenates were incubated with various concentrations of [^{3}H]-granisetron at 37°C for 60 min. Specific binding was determined as the difference between total and non-specific binding (defined by the presence of ondansetron; 10 μM). The results of a representative experiment depicting the total (▲), specific (■) and non specific (▼) binding of [^{3}H]-granisetron are illustrated. Total and non-specific binding points represent the mean of triplicate determinations. (b) Kinetic analysis of [^{3}H]-granisetron binding. In the association reaction (upper panel), membranes were incubated with [^{3}H]-granisetron (1 nM), in the presence, or absence, of ondansetron (10 nM), in a total volume of 30 ml at 37°C for 60 min; 1 ml aliquots were removed for analysis at set time points. In the dissociation reaction (lower panel), membranes were pre-incubated as described for association studies. After 60 min dissociation was initiated by the addition of ondansetron (10 μM) and allowed to proceed for a further 60 min; 1 ml aliquots were processed at set time points. The data are expressed as specific binding versus incubation time. Each graph is representative of three independent experiments.

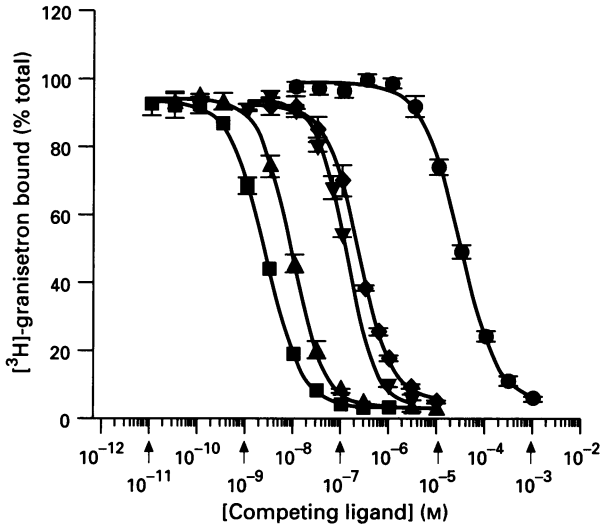

Figure 3 Competition for [^{3}H]-granisetron binding to the h5-HT$_3$R-A$_S$ homo-oligomer by 5-HT$_3$ receptor agonists and antagonists. Membrane homogenates prepared from HEK293 cells stably expressing the h5-HT$_3$R-A$_S$ cDNA were incubated with [^{3}H]-granisetron (1.0 nM) in the presence of various concentrations of granisetron (■), ondansetron (▲), *m*-chlorophenylbiguanide (▼), 5-HT (◆) and (+)-tubocurarine (●). Each data point represents the results of 3 experiments (mean ± s.e. mean), expressed as a percentage of total [^{3}H]-granisetron bound versus the concentration of the competing drug. The IC$_{50}$ for each agent was determined with a computer assisted iterative curve fitting program (four parameter logistic fit). Inhibitor constants (pK_i), derived from the Cheng-Prusoff relationship are summarized in Table 1.

Table 1 Binding parameters of the h5-HT$_3$R-A$_S$ expressed in HEK 293 cells

Compound	pK_i (nM)	n_H
Granisetron	8.84±0.04	1.13±0.04
Ondansetron	8.31±0.06	1.25±0.07
m-CPBG	7.17±0.02	1.3±0.10
5-HT	6.91±0.03	1.26±0.11
2-Me-5-HT	6.65±0.08	1.26±0.09
Metoclopramide	6.45±0.03	1.07±0.08
PBG	5.62±0.03	1.34±0.06
Cocaine	5.40±0.06	1.10±0.05
(+)-Tubocurarine	4.80±0.03	1.09±0.09
Cyproheptadine	14±1%*	–
Atropine	69±1%*	–
8-OH-DPAT	71±2%*	–
5-Methoxytryptamine	74±2%*	–
Dopamine	89±1%*	–
(−)-Propranolol	91±3%*	–
Noradrenaline	91±3%*	–
Phentolamine	99±1%*	–
Yohimbine	102±1%*	–
Methysergide	106±2%*	–
Ketanserin	109±2%*	–

Data are the mean±s.e.mean of values from three to six observations. For each compound, the pK_i and Hill coefficient (n_H) are given except for those denoted with an asterisk where the value indicates the percentage specific binding remaining in the presence of the competing drug at 10 μM.

have not been examined in human tissue. However, in membranes prepared from rat brain cortes (Nelson & Thomas, 1989), N1E-115 neuroblastoma cells (Morain *et al.*, 1994) and NG108-15 neuroblastoma × glioma cells (Wong *et al.*, 1995), the kinetics of [^{3}H]-granisetron binding were monophasic and yielded estimates of k_{+1} and k_{-1} comparable to those obtained in the present study, allowing for the lower temperature (room; 23–25°C) at which those assays were performed. By contrast, although the association of [^{3}H]-granisetron with the mouse homologue of the 5-HT$_3$R-A$_S$ expressed in HEK 293 cells is apparently monophasic, dissociation is clearly biphasic (Steward *et al.*, 1995). It should be noted, however, that the latter experiments were conducted at 4°C and that another study performed at 25°C found no evidence for a biphasic dissociation of [^{3}H]-granisetron from HEK 293 cells expressing the mouse 5-HT$_3$R-A (Bonhaus *et al.*, 1995). Thus it is possible that a fast component of dissociation was undetected in the present experiments conducted at 37°C. Nonetheless the kinetically derived K_D for [^{3}H]-granisetron (0.45 nM) was in tolerable agreement with the value (1.4 nM) found in saturation and competition studies.

In agreement with previous studies upon mouse recombinant 5-HT$_3$R-A$_S$ receptors expressed in HEK 293 cells (Gill *et al.*, 1995), the stable transfection of this cell line with the cDNA encoding the h5-HT$_3$R-A$_S$ resulted in expression of large inward current responses to locally applied 5-HT which reversed in sign at a holding potential close to 0 mV. Granisetron, at a concentration (i.e. 1 nM) approximating to the K_D, produced an almost complete suppression of the response to 5-

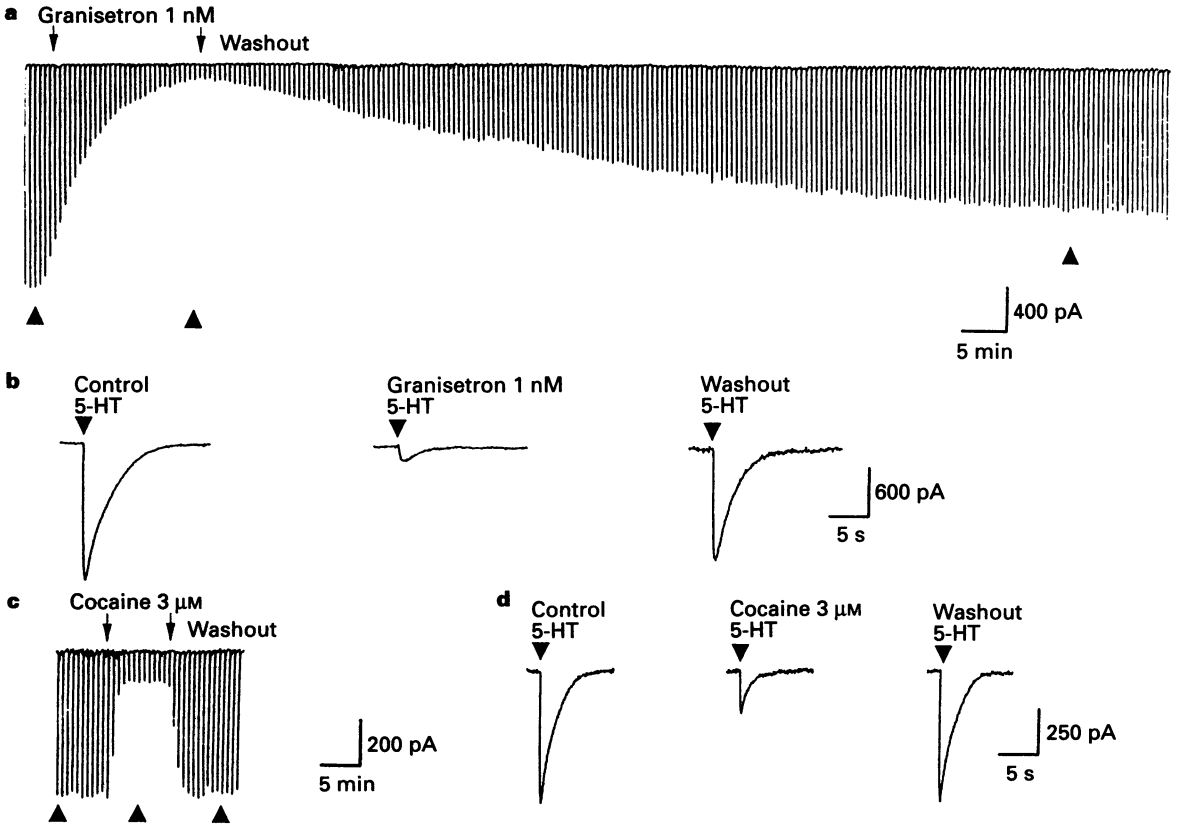

Figure 4 5-HT-induced inward current responses recorded from HEK 293 cells stably transfected with the h5-HT$_3$R-A$_S$ cDNA are antagonized by granisetron. (a) Trace illustrating, on a slow time base, inward current responses in response to the repetitive (0.05 Hz) applications of 5-HT (10 μM) by local pressure ejection (30 ms; 1.4×10^5 Pa). Bath applied granisetron (1 nM) exerts a virtually complete blockade of the response that is slow to develop and reverse. (b) 5-HT-induced currents, sampled at the points indicated by solid triangles in (a), are illustrated on an expanded time-base. (c and d) Blockade of the 5-HT-induced current by cocaine (3 μM) illustrated on slow (c) and expanded (d) time bases. Note that blockade by cocaine develops and reverses comparatively rapidly. All records were obtained from the same HEK 293 cell voltage-clamped at a holding potential of −60 mV. Agonist-evoked currents were recorded at ambient temperature (20°C).

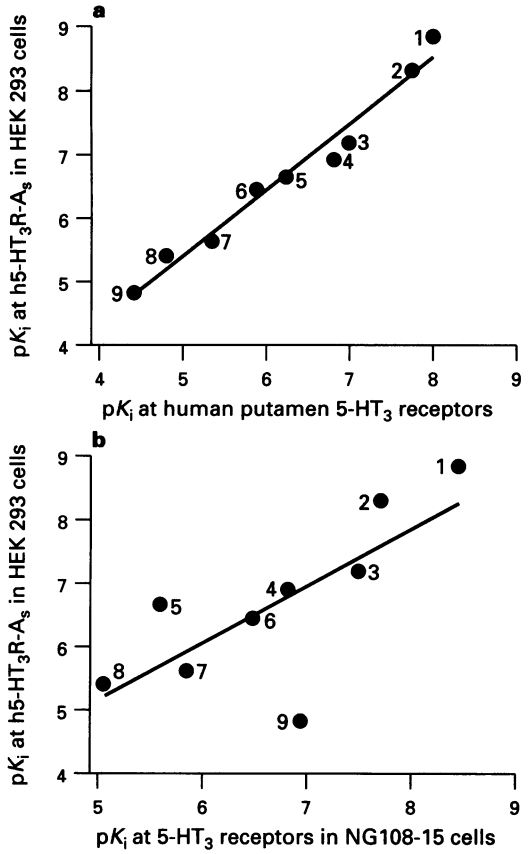

Figure 5 Correlation between the affinities of ligands for the displacement of specific [^{3}H]-granisetron binding to the homo-oligomeric cloned h5-HT$_3$R-A$_S$ receptor stably expressed in HEK293 cells and 5-HT$_3$ receptors native to the human and mouse (Bufton *et al.*, 1993). (a) A comparison of the pK_i values of agonists and antagonists observed for h5-HT$_3$R-A$_S$ (ordinate) and 5-HT$_3$ receptors expressed in membranes prepared from human putamen post-mortem tissue (abscissa). The line was fitted to the data points by least squares linear regression analysis and yields a correlation coefficient (r) of 0.99 and a slope of 1.03. (b) A comparison of the pK_i values of agonists and antagonists for h5-HT$_3$R-A$_S$ (ordinate scale) and 5-HT$_3$ receptors expressed in membranes prepared from the murine hybridoma cell line NG108-15 (abscissa) yielded a correlation coefficient (r) of 0.74 and a slope of 0.90. The data were analysed by least squares linear regression. The individual data points in each graph correspond to (1) granisetron; (2) ondansetron; (3) *m*-CPBG; (4) 5-HT; (5) 2-Me-5-HT; (6) metoclopramide; (7) PBG; (8) cocaine and (9) (+)-tubocurarine.

HT which was slow to develop and reverse. The apparently greater potency of granisetron in the electrophysiological assay was not unexpected, since equilibrium between agonist and antagonist binding cannot be obtained during the limited duration of the inward current response (see below and review by Peters *et al.*, 1994). The time course for reversal of antagonism by granisetron, which was studied in detail in two cells only, was considerably slower than that found in binding assays. The lower temperature at which the electrophysiological experiments were conducted may have contributed to this discrepancy, although other dissimilarities between the two assay systems might also be pertinent.

The pharmacological properties of the [^{3}H]-granisetron-labelled recombinant receptor appear very similar to those established for 5-HT$_3$ receptors identified in post-mortem human brain tissue utilizing the same radioligand (Bufton *et al.*, 1993). Thus, in competition studies, the rank order of potency with which 9 compounds competed for the [^{3}H]-granisetron-labelled recombinant receptor was identical to that found for the 5-HT$_3$ receptor in membrane homogenates of the human putamen (Bufton *et al.*, 1993). The pK_i values of the competing ligands were, in general, slightly larger than those reported by Bufton *et al.* (1993; see Table 1), but overall, there is good quantitative agreement between the pK_i values determined in the present study and those found for 5-HT$_3$ receptors labelled with either [^{3}H]-zacopride (Barnes *et al.*, 1989) or [^{3}H]-LY278584 (Abi-Dargham *et al.*, 1993) in membrane homogenates of human amygdala, caudate and hippocampus.

The pharmacological profile of the h5-HT$_3$R-A$_S$ subunit expressed in *Xenopus laevis* oocytes was recently examined utilizing voltage-clamp techniques (Belelli *et al.*, 1995). Although the competition studies reported here indicate a similar profile, a comparison of antagonist K_i values with functionally determined IC_{50} values suggests antagonist potency to be greater in the electrophysiological assay. Conversely, agonist K_i values consistently provide apparent affinities higher than those inferred from the EC_{50} values associated with agonist-evoked inward current responses in oocytes (Belelli *et al.*, 1995). A major confounding factor in such a comparison is the development of receptor desensitization, which in electrophysiological assays curtails the measured response before equilibrium between agonist and antagonist binding can be attained (reviewed by Peters *et al.*, 1994). Such a phenomenon would be expected to yield exaggerated estimates of antagonist potency in functional studies. However, with respect to agonists, binding assays may detect a desensitized state of the receptor that demonstrates higher affinity towards agonists than the conducting state of the receptor assayed electrophysiologically (Sepúlveda *et al.*, 1991; Boess *et al.*, 1992). Aside from the influence of desensitization, a recent study performed upon both recombinant and native receptors found evidence for allosteric interactions between agonist and antagonist binding within the 5-HT$_3$ receptor complex that further complicates attempts to relate the results of radioligand binding and functional studies (Bonhaus *et al.*, 1995).

5-HT$_3$ receptors endogenous to central and peripheral neurones exhibit clear species differences in their pharmacological profile (see Introduction and Figure 5b). Such differences are pronounced in the case of the receptor expressed by guinea-pig tissues, which in comparison to that present in several other species, demonstrates a generalized reduction in affinity toward most ligands (Butler *et al.*, 1990; Wong *et al.*, 1993b). However, dissimilarities in the properties of 5-HT$_3$ receptors of the mouse, rat and rabbit can also be detected with compounds that include (+)-tubocurarine (e.g. Newberry *et al.*, 1991; Peters *et al.*, 1991) and arylbiguanides (e.g. Kilpatrick *et al.*, 1991; Wong *et al.*, 1993b). In the present study, the binding of (+)-tubocurarine was of much lower affinity than that determined for the [^{3}H]-granisetron labelled mouse 5-HT$_3$R-A expressed in HEK 293 cells (200 fold, Bonhaus *et al.*, 1995). This result is in qualitative agreement with recent functional studies which have demonstrated an approximately 1800 fold discrepancy in the potency of (+)-tubocuraine as an antagonist of 5-HT evoked currents mediated by mouse and human 5-HT$_3$R-A$_S$ receptors expressed in *Xenopus laevis* oocytes (Belelli *et al.*, 1995). Similarly, the affinity of (+)-tubocurarine was considerably lower than that found for 5-HT$_3$ receptors native to mouse cortex, ileum or colon (40–320 fold; Bonhaus *et al.*, 1993; Perren *et al.*, 1995), rat cortex (68–163 fold; Kilpatrick *et al.*, 1990; Wong *et al.*, 1993b), pig cortex (15 fold; Fletcher & Barnes, 1995 and N.M. Barnes, personal communication) or rabbit ileum (23 fold; Wong *et al.*, 1993b), but approximated to that found for the receptor present in guinea-pig ileum ($\approx$2 fold difference; Wong *et al.*, 1993b). A specific tryptophan residue (W66) within the N-terminal domain of the mouse 5-HT$_3$R-A has recently been identified as a potential component of the binding site for (+)-tubocurarine and other antagonists (Schulte *et al.*, 1995). However, both the tryptophan residue and the amino acids which immediately flank it are conserved across all 5-HT$_3$R-A species homologues identified to date and are thus unlikely to contribute to the pronounced species-dependent affinity of (+)-tubocurarine.

K_i values determined for PBG and *m*-CPBG were similar to those found in mouse and rabbit tissues (Kilpatrick *et al.*,

1991; Bonhaus *et al.*, 1993; Perren *et al.*, 1995) and intermediate to the low and high K_i values reported for rat (Kilpatrick *et al.*, 1990; Bonhaus *et al.*, 1993) and guinea-pig (Wong *et al.*, 1993b) 5-HT$_3$ receptors respectively. Interestingly, the affinity of *m*-CPBG for the h5-HT$_3$R-A$_S$ expressed stably in HEK293 cells and transiently in COS-1 cells (Miyake *et al.*, 1995) is approximately 100 fold lower than that reported for the rat 5-HT$_3$R-A$_S$ subunit (Miyake *et al.*, 1995). A similar, though less pronounced trend, is apparent for other agonists including 5-HT and 2-methyl-5-HT and 5-HT. Studies with a range of recently described di- and tri-chloro derivatives of PBG with improved affinity for murine 5-HT$_3$ receptors (Morain *et al.*, 1994) may prove useful in further dissecting species differences in the properties of both recombinant and native receptors.

In summary, the pharmacological profile of the h5-HT$_3$R-A$_S$ stably expressed in HEK 293 cells is dissimilar from that of the receptor expressed by routinely studied animal species, but appears to be representative of the receptor endogenous to human brain. The 5-HT$_3$R-A subunits cloned from mouse, rat and human tissues demonstrate a high degree of sequence homology (see Belelli *et al.*, 1995; Miyake *et al.*, 1995) and within the extracellularly located N-terminal domain, which contains the ligand binding site(s) (Eiselé *et al.*, 1993), there are few amino acids which are unique to a particular species homologue. It can be anticipated that sequence comparisons will provide important clues for studies employing site-directed mutagenesis which aim to identify binding domains upon the 5-HT$_3$ receptor. In the meantime, the availability of a stably transfected cell line expressing the h5-HT$_3$R-A$_S$ at high density should prove useful for the further characterization of the pharmacological and biophysical properties of the human 5-HT$_3$ receptor.

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