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European Journal of Pharmacology 320 (1997) 267–275

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Characterisation of a cloned human 5-HT_{1A} receptor cell line using [^{35}S]GTPγS binding

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Received 27 August 1996; revised 11 November 1996; accepted 15 November 1996

Abstract

Compound potencies and efficacies depend upon receptor reserve and hence estimating this parameter in assay systems allows for a more meaningful interpretation of the data generated. This study describes a method whereby the degree of receptor reserve, with respect to 5-hydroxytryptamine (5-HT), was determined for a HeLa cell line expressing the human 5-HT_{1A} receptor using the agonist-induced [^{35}S]guanosine 5′[γ-thio]triphosphate ([^{35}S]GTPγS) binding assay, followed by a comparison of the potencies and relative efficacies of several compounds. Following irreversible antagonism with benextramine 5-HT yielded a pK_A of 7.3, compared with a pK_{obs} of 8.4 from saturation analysis, indicating the presence of high and low affinity state receptors. A 20% receptor occupancy elicited a half-maximal functional response consistent with the presence of receptor reserve. 5-HT, 5-carboxamidotryptamine (5-CT), 8-hydoxy-dipropylamino-tetralin (8-OH-DPAT), 5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indole (RU24969), buspirone, gepirone, mesulergine and sumatriptan were equally efficacious. 1-(2-Methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine (NAN 190) displayed reduced relative efficacy and methiothepin inverse agonism.

Keywords: [^{35}S]GTPγS binding assay; 5-HT_{1A} receptor, human; Receptor reserve

1. Introduction

Anomalous findings regarding the efficacy and potency of 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor selective compounds have, in the past, led to the suggestion of the presence of multiple 5-HT_{1A} receptor subtypes (Dumuis et al., 1988). An alternative explanation, however, is the presence of varying degrees of receptor reserve in differing tissues. For example, the findings of Meller et al. (1990) and more recently Yocca et al. (1992), indicate a lack of receptor reserve in rat hippocampus compared with a large receptor reserve for somatodendritic 5-HT_{1A} receptors in raphe nuclei. This offers an explanation for the partial agonism seen with the anxiolytic/antidepressant compounds buspirone, gepirone and ipsapirone (Rodgers and Cooper, 1991) in biochemical (Bockaert et al., 1987; Meller et al., 1990) and electrophysiological (Sprouse and Aghajanian, 1988; Martin and Mason, 1987) models of postsynaptic hippocampal 5-HT_{1A} receptor function compared with the full agonism seen using raphe nuclei preparations, an area where pre-synaptic 5-HT_{1A} receptors are located (Sprouse and Aghajanian, 1987, 1988; Meller et al., 1990). Likewise, compounds with lower intrinsic activity, e.g., 8-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-8-azaspirol (4.5)-decane-7,9-dione (BMY 7378), 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine (NAN 190) and 4-(benzodioxan-5-yl)1-(indan-2-yl)piperazine (S 15535) (Glennon and Dukat, 1991) act as agonists and antagonists at pre- and postsynaptic receptors, respectively (Hjorth and Sharp, 1990; Sharp et al., 1990; Millan et al., 1993).

The advent of molecular cloning techniques and the availability of cell lines stably transfected with various neurotransmitter receptors offer powerful tools for investigating the biochemical mechanisms whereby their signals are transduced. Such techniques have allowed for the determination of affinity, potency and efficacy values of

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PII S0014-2999(96)00914-4

compounds at the cloned rat and human 5-HT_{1A} receptors stably expressed in HeLa cells (Fujiwara et al., 1993; Pauwels et al., 1993; Fargin et al., 1989). In addition, however, it was soon established that the level of expression of the cloned human 5-HT_{1A} receptor within a particular cell line, had a marked effect on the ability of agonists to activate secondary messenger systems. Hence, 5-HT was equipotent in inhibiting forskolin-stimulated adenylyl cyclase in HA6 cells (HeLa cells with higher levels of 5-HT_{1A} receptor expression) and HA7 cells (HeLa cells with lower levels of 5-HT_{1A} receptor expression) whereas 5-HT was 10–100-fold less potent in stimulating phospholipase C in HA7 cells (Boddeke et al., 1992; Hoyer and Boddeke, 1993). Further complications are the potential of altered signal transduction depending on the cellular content of the host cell line, in terms of their complement of endogenous G proteins, signaling enzymes and effector systems. Hence, whereas similar coupling patterns are seen with human 5-HT_{1A} receptors expressed in fibroblast CHO and epithelial HeLa cells (Boddeke et al., 1992; Raymond et al., 1992), rat 5-HT_{1A} receptors expressed in GH4C1 pituitary and mouse fibroblast Ltk^- cells display strikingly different patterns of coupling (Lui and Albert, 1991).

A full in vitro characterisation of 5-HT_{1A} selective compounds, in terms of their relative efficacy and potency offers valuable information in attempting to predict their in vivo properties. In order to exploit the availability of cloned human receptors stably expressed in cell lines, the cell lines themselves must first be characterised in terms of the receptor coupling efficiency. Also the degree of receptor reserve, with respect to the endogenous ligand, offers additional information useful in the interpretation of the relative efficacies of novel compounds.

Here we describe, using the guanine nucleotide protein (G protein)-coupled receptor functional assay, the agonist-induced [^{35}S]guanosine 5′[γ-thio]triphosphate ([^{35}S]GTPγS) binding assay (Hilf et al., 1989; Lazareno and Birdsall, 1993), an efficient means of characterising the cloned human 5-HT_{1A} receptor stably expressed in HeLa cells (HA6) and the subsequent use of this cell line to compare the relative efficacies and potencies of 5-HT_{1A} receptor agents. The degree of receptor reserve, with respect to the endogenous agonist 5-HT, was determined by comparing the ability of 5-HT to induce [^{35}S]GTPγS binding before and after pre-treatment of the cells with the alkylating agent benextramine (Furchgott, 1966). The assay system has then been employed to compare several selective and non-selective 5-HT_{1A} receptor agents in terms of their relative efficacies and potencies. One compound, the 5-HT_1/5-HT_2 receptor non-selective agent methiothepin, which had previously been thought of as a silent antagonist has been shown to display inverse agonism in this system. Methiothepin has previously been shown to be an inverse agonist at cloned human 5-HT_{1D_α} and 5-HT_{1D_β} receptors stably expressed in CHO cells (Thomas et al., 1995).

2. Materials and methods

2.1. [^{3}H]5-HT and [^{3}H]8-hydroxy-dipropylamino-tetralin ([^{3}H]8-OH-DPAT) displacement studies and [^{3}H]5-HT saturation analysis

HeLa cells stably expressing the cloned human 5-HT_{1A} receptor (HA6, Fargin et al., 1989) were grown, to confluence, harvested in phosphate buffered saline and stored at −70°C. On the day of assay cells were thawed, homogenised in 10–15 volumes of ice-cold 50 mM Tris-HCl (pH 7.7 at room temperature) with a Kinematica polytron (setting 5, 10 s) and centrifuged at 48 000 × *g*, 4°C for 11 min. The resulting supernatant was discarded and the pellet resuspended in the same volume of ice-cold Tris-HCl before being incubated at 37°C for 10 min, to remove any endogenous 5-HT, and recentrifuged at 48 000 × *g*, 4°C for a further 11 min. The final pellet was then resuspended in 50 mM Tris assay buffer containing 0.1% ascorbate, 10 μM pargyline and either 5.7 mM $CaCl_2$ ([^{3}H]8-OH-DPAT studies) or 4 mM $CaCl_2$ ([^{3}H]5-HT studies), pH 7.7 at room temperature, to give a washed wet weight-volume ratio of 1 : 250. This resulted in a final protein concentration of 10–15 μg per tube. The concentrations of $CaCl_2$ used in the [^{3}H]8-OH-DPAT and [^{3}H]5-HT displacement studies were chosen on historical grounds. A comparison of the affinity values for a wide range of compounds, determined under both assay conditions, are in excellent agreement (see below). This indicates that the differing $CaCl_2$ concentrations have no effect on the receptor affinity state and that the results generated using the two assays are directly comparable.

All assays were carried out in duplicate on 3–4 separate occasions. Test drug or buffer was incubated with 500 μl membrane, 4.0 nM [^{3}H]5-HT/1.5 nM [^{3}H]8-OH-DPAT (displacement studies) or 0.1–40 nM [^{3}H]5-HT (saturation studies) in a final assay volume of 1 ml, at 37°C in a shaking water bath. 5-HT (10 μM) was used to define non-specific binding. The incubation was started by addition of the membrane suspension and was terminated, after 20 min for the [^{3}H]8-OH-DPAT studies and 30 min for the [^{3}H]5-HT studies, by rapid filtration over GF/B filters using a Brandel cell harvester. Each assay tube was washed twice with 4 ml of ice-cold Tris-HCl. Filters had previously been soaked in 0.3% polyethylenimine-0.5% Triton X-100 to reduce non-specific binding. Radioactivity was counted by liquid scintillation spectrometry (45–55% efficiency).

2.2. Agonist-induced [^{35}S]GTPγS binding

HeLa cells stably expressing cloned human 5-HT_{1A} receptors were grown and harvested as described above. Cell membranes were prepared and the assay carried out essentially as described by Lazareno and Birdsall (1993). Cells were homogenised in ice-cold 20 mM Hepes buffer

containing 10 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4 at room temperature) using a Kinematica polytron (setting 5, 10 s) and centrifuged at 40 000 × *g*, 4°C for 15 min. The pellet was resuspended in ice-cold 20 mM Hepes buffer containing 0.1 mM EDTA (pH 7.4 at room temperature) and recentrifuged at 40 000 × *g*, 4°C for 15 min. For the receptor reserve studies cells were pre-treated prior to being harvested, with the alkylating agent benextramine (3, 6 and 10 μM) for 60 min. The final pellet was resuspended in 20 mM Hepes buffer containing 100 mM NaCl, 10 mM $MgCl_2$, 0.1% ascorbate and 10 μM pargyline, pH 7.4 at room temperature.

All assays were carried out in duplicate on 3–4 separate occasions. Membranes (5 mg wet weight (40–80 μg protein), 1–10 mg wet weight (8–150 μg protein) for optimisation studies) were incubated with GDP (30 μM, 1–100 μM for optimisation studies) and test drug (plus antagonist or second agonist when tested) for 20 min at 30°C in a volume of 900 μl and then transferred to ice for 15 min. [^{35}S]GTPγS (100 pM) was added to all tubes giving a final assay volume of 1 ml and the tubes incubated for a further 30 min at 30°C. The incubation was terminated by filtering over GF/B filters using a Brandel cell harvester and the filters were washed once with 5 ml of water. Radioactivity was counted by liquid scintillation spectrometry at an efficiency of > 90%.

2.3. Data analysis

Radioligand binding displacement and saturation curves, and agonist-induced [^{35}S]GTPγS dose–response curves were analysed by non-linear, least squares regression analysis using an iterative curve fitting routine (Marquardt-Levenberg method) provided by the data manipulation software RS/1 (Software Products Corporation, Cambridge, MA, USA).

In all radioligand binding studies data represents specific binding only (total-non-specific). Displacement and composite saturation curves were analysed based on the assumption of a one-site model followed by that for a two-site model. Improvement of the fit by a two-site model was tested using the partial *F*-test procedure as described by DeLean et al. (1978). A two-site model was only accepted if the probability of the models being the same was less than 1:20 ($P < 0.05$). The affinity values of competing compounds in the displacement studies are expressed as pK_i values ($-\log_{10} K_i$) where the K_i value is calculated from the IC_{50} (concentration of drug which inhibits 50% binding) value using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) as follows: $IC_{50} = K_i (1 + S/K_d)$ where S is the radioligand concentration and K_d is the ligand dissociation constant (4 nM for both [^{3}H]8-OH-DPAT and [^{3}H]5-HT). The affinity value for [^{3}H]5-HT is expressed as pK_{obs} ($-\log K_{obs}$). Data are expressed as means ± S.E.M. from 3–4 experiments.

Background filter counts, that is residual radioactivity bound to the filter in the absence of membrane, was subtracted from each sample count for the agonist-induced [^{35}S]GTPγS binding experiments. Test results were then represented as percentage increase in binding above basal, i.e., that seen in the absence of test compound, and dose–response curves were plotted and analysed. Potency values are expressed as pD_2 values ($-\log_{10}$ concentration of agonist required to give 50% of its own maximal stimulation). The maximal stimulation (E_{max}) achieved for each drug was expressed as a percentage of the maximal 5-HT response. For the benextramine study, the stimulation of [^{35}S]GTPγS binding was expressed as fmol bound/mg protein at each concentration of 5-HT tested. Data are expressed as means ± S.E.M. from 3–4 experiments.

The concentration of 5-HT which produced half of its maximal response in the agonist-induced [^{35}S]GTPγS binding assay was determined in the absence and presence of 30 and 100 nM methiothepin. Dose ratios (DR) were determined at the EC_{50} values of 5-HT. The apparent pA_2 for methiothepin was determined using the equation: apparent $pA_2 = \log_{10} (DR - 1) - \log_{10}$ (molar concentration of methiothepin).

The agonist-induced [^{35}S]GTPγS binding assay was used to determine the K_A value for 5-HT by plotting the reciprocal values of equipotent concentrations of 5-HT before (1/[A]) and after (1/[A′]) pre-treatment with benextramine, ensuring that the concentration of benextramine used caused a depression in the maximal 5-HT response. This resulted in a straight line graph from which the K_A was determined using the equation K_A = (slope − 1)/intercept (Furchgott, 1966). The fractional receptor occupancy, at varying agonist concentrations, was then calculated using the equation, receptor occupancy = $([A]/([A] + K_A)) \times 100$, where [A] = fixed agonist concentration and K_A = dissociation constant for the agonist. The receptor occupancy was then plotted against fractional response in order to determine the degree of receptor reserve.

2.4. Materials

5-Hydroxy[^{3}H]tryptamine creatinine sulphate (10–20 Ci/mmol) and 8-hydroxy-[^{3}H]DPAT (160–240 Ci/mmol) were purchased from Amersham International (Amersham, UK), and guanosine 5′[γ-thio] triphosphate, [^{35}S] ([^{35}S]GTPγS) (1000–1500 Ci/mmol) from Du Pont NEN Research Products (Wilmington, DE, USA). The following compounds were purchased from Research Biochemicals (Natick, MA, USA): 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide (NAN 190), (±)-8-hydroxy-dipropylamino-tetralin HBr (8-OH-DPAT), metergoline, buspirone HCl, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride (WB 4101), methiothepin mesylate, pindolol, rauwolscine HCl, cyproheptadine HCl, mesulergine HCl and 3-tropanyl-3,5-dihydrchlorobenzoate (MDL 72222). Guanosine 5′-diphosphate (GDP), 5-hydroxytryptamine creatinine sulphate complex and 3-hydroxytyra-

mine HCl (dopamine) were purchased from Sigma (St. Louis, MO, USA) and noradrenaline from Aldrich (Milwaukee, WI, USA). 5-carboxamidotryptamine (5-CT), 3-(1,2,5,6-tetrahydropyrid-4-yl)-pyrrolo[3,2-b]pyrid-5-one) (CP 93,129) and sumatriptan were synthesised at Merck Sharp & Dohme. Gepirone, ipsapirone and 5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indole (RU24969) were kind gifts from Bristol-Myers, Troponwerke and Roussel Uclaf, respectively.

HeLa cells stably expressing the cloned human 5-HT_{1A} receptor were purchased from the Howard Hughes Medical Institute, Duke University (Durham, NC, USA).

3. Results

3.1. [^{3}H]8-OH-DPAT and [^{3}H]5-HT radioligand binding studies

Specific [^{3}H]8-OH-DPAT binding typically accounted for 94–96% of the total binding whereas specific [^{3}H]5-HT typically accounted for 67–95% of total binding. Approximately 4300 fmol/mg protein of [^{3}H]8-OH-DPAT and 4100 fmol/mg protein of [^{3}H]5-HT was bound in the respective displacement studies, and 290–10 900 fmol/mg protein [^{3}H]5-HT in the saturation studies. In all experiments binding to filters was less than 10% of the specific binding (data not shown).

Computer-assisted iterative curve fitting analysis consistently gave monophasic displacement and saturation curves.

[^{3}H]5-HT labeled, with high affinity, an apparent homogeneous population of binding sites in HeLa cells stably transfected with cloned human 5-HT_{1A} receptors (HA6). Binding was saturable and yielded a pK_{obs} of 8.39 ± 0.02 and a B_{max} of $10\,000 \pm 1400$ fmol/mg protein (mean $\pm$ S.E.M., $n = 3$).

Displacement studies were carried out using [^{3}H]5-HT at its K_d concentration (4 nM) and [^{3}H]8-OH-DPAT at approximately half its K_d concentration (1.5 nM). The pK_i values for 20 compounds are given in Table 1. A comparison of the pK_i values generated yielded a correlation coefficient $r = 0.99$, significance level $P = 0.001$.

3.2. Agonist-induced [^{35}S]GTPγS binding studies

5-HT caused a dose-dependent increase in [^{35}S]GTPγS binding at the cloned human 5-HT_{1A} receptor, stably expressed in HeLa cells. Initial experiments varying GDP and membrane concentrations were carried out to determine optimal conditions for 5-HT-induced stimulation of [^{35}S]GTPγS binding. As can be seen in Fig. 1a, in the presence of 5 mg wet weight membrane, the addition of 1,

Table 1
A comparison of binding affinities for a series of standard compounds at the cloned human 5-HT_{1A} receptor stably expressed in HeLa cells labeled with [^{3}H]5-HT and [^{3}H]8-OH-DPAT

Compound	[^{3}H]5-HT		[^{3}H]8-OH-DPAT	
	$pK_i \pm$ S.E.M.	$n_H \pm$ S.E.M.	$pK_i \pm$ S.E.M.	$n_H \pm$ S.E.M.
5-CT	9.40 ± 0.09	1.08 ± 0.16	9.37 ± 0.05	0.78 ± 0.19
NAN 190	8.91 ± 0.10	0.89 ± 0.10	8.72 ± 0.05	1.13 ± 0.14
8-OH-DPAT	8.76 ± 0.11	0.84 ± 0.09	8.70 ± 0.05	0.96 ± 0.10
Metergoline	8.49 ± 0.02	0.82 ± 0.02	7.98 ± 0.12	1.03 ± 0.09
5-HT	8.38 ± 0.09	1.13 ± 0.06	8.35 ± 0.07	0.85 ± 0.09
WB 4101	8.22 ± 0.08	0.95 ± 0.02	8.16 ± 0.08	0.99 ± 0.06
RU24969	8.07 ± 0.09	0.90 ± 0.08	8.07 ± 0.10	0.89 ± 0.04
Ipsapirone	8.01 ± 0.17	0.94 ± 0.10	7.88 ± 0.09	0.97 ± 0.08
Methiothepin	7.76 ± 0.12	0.84 ± 0.05	7.49 ± 0.08	0.79 ± 0.05
Buspirone	7.52 ± 0.05	0.78 ± 0.04	7.48 ± 0.12	0.82 ± 0.02
Pindolol	7.46 ± 0.09	0.96 ± 0.22	7.41 ± 0.17	0.97 ± 0.20
Rauwolscine	7.36 ± 0.12	0.76 ± 0.08	7.22 ± 0.13	0.98 ± 0.05
Gepirone	7.00 ± 0.15	0.96 ± 0.17	6.76 ± 0.05	0.84 ± 0.05
Cyproheptadine	6.98 ± 0.15	0.89 ± 0.16	6.89 ± 0.17	0.90 ± 0.06
Mesulergine	6.54 ± 0.15	0.73 ± 0.14	6.17 ± 0.02	1.00 ± 0.15
Sumatriptan	6.53 ± 0.06	1.08 ± 0.04	6.40 ± 0.09	0.84 ± 0.10
CP 93,129	5.77 ± 0.02	0.98 ± 0.20	5.52 ± 0.04	1.12 ± 0.06
MDL 72222	< 5.0	–	< 5.0	–
Noradrenaline	< 5.0	–	< 5.0	–
Dopamine	< 5.0	–	< 5.0	–

Affinity (pK_i) and n_H values for compounds at the [^{3}H]5-HT and [^{3}H]8-OH-DPAT recognition sites in membranes prepared from HeLa cells stably expressing the cloned human 5-HT_{1A} receptor. The pK_i values generated using [^{3}H]5-HT are in excellent agreement with the pK_i values generated using [^{3}H]8-OH-DPAT (correlation coefficient $r = 0.99$, significance level $P = 0.001$). Results are arithmetic means $\pm$ S.E.M. of 3–4 experiments. Data yielded displacement curves which were fitted best by a one site model ($P > 0.05$, partial F-test). For details of experimental conditions, see Section 2 and Section 2.3.

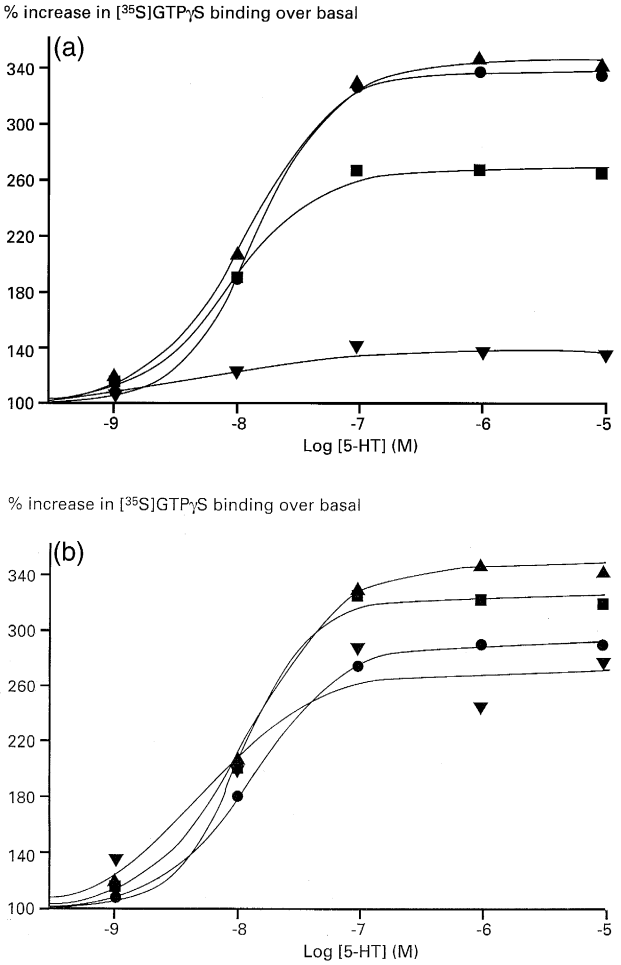

Fig. 1. 5-HT-induced [^{35}S]GTPγS binding dose–response curves carried out at the cloned human 5-HT$_{1A}$ receptor stably expressed in HeLa cells in the presence of increasing concentrations of GDP and of membrane. (a) The effects of increasing GDP concentrations, 1 μM (▼), 10 μM (■), 30 μM (▲) and 100 μM (●) using 5 mg wet weight of membrane. (b) The effect of increasing membrane concentrations, 1 mg (▼), 3 mg (■), 5 mg (▲) and 10 mg (●) wet weight using 30 μM GDP. Each curve represents a composite of 3 experiments. Optimal assay conditions were 30 μM GDP and 5 mg wet weight of membrane and subsequent assays were carried out using these conditions. For details of experimental conditions, see Section 2 and Section 2.3.

10 and 30 μM GDP resulted in a dose-dependent increase in the maximal response elicited by 5-HT (131 ± 24, 261 ± 35, 336 ± 30% increase in [^{35}S]GTPγS binding over basal (mean ± S.E.M., $n = 3$), respectively). This maximal response was not improved in the presence of 100 μM GDP (330 ± 49% increase in [^{35}S]GTPγS binding over basal (mean ± S.E.M., $n = 3$)) and therefore 30 μM GDP was used in all further experiments. Studies to investigate the effects of increasing concentrations of membrane, in the presence of 30 μM GDP, again yielded a dose-dependent increase in the maximal response at 1, 3 and 5 mg wet weight membrane (271 ± 58, 312 ± 56 and 336 ± 30% increase in [^{35}S]GTPγS binding over basal (mean ± S.E.M., $n = 3$), respectively) as shown in Fig. 1b. This maximal response was depressed with 10 mg wet weight membrane (284 ± 38% increase in [^{35}S]GTPγS binding over basal (mean ± S.E.M., $n = 3$) and hence 5 mg wet weight membrane was used in all further experiments.

Basal and maximal 5-HT-stimulated [^{35}S]GTPγS binding levels were 81 ± 12 and 210 ± 17 fmol/mg protein,

Table 2
Potency and efficacy values for a series of standard compounds using [^{35}S]GTPγS binding to cloned human 5-HT$_{1A}$ receptors stably expressed in HeLa cells

Compound	pD_2 ± S.E.M.	E_{max} ± S.E.M.
5-HT	7.90 ± 0.08	100
5-CT	8.82 ± 0.16	99 ± 5
8-OH-DPAT	8.34 ± 0.03	92 ± 2
RU24969	7.88 ± 0.05	96 ± 6
Buspirone	7.11 ± 0.02	86 ± 3
Gepirone	6.54 ± 0.06	83 ± 8
Mesulergine	5.91 ± 0.03	86 ± 1
Sumatriptan	5.81 ± 0.06	100 ± 6
NAN 190	8.51 ± 0.13	30 ± 4
Methiothepin	–	46 ± 19 [a]

[a] Percentage inhibition of basal response. Potency (pD_2) and relative efficacy values (E_{max}) for a series of compounds in the agonist-induced [^{35}S]GTPγS binding assay carried out in membranes prepared from HeLa cells stably expressing the cloned human 5-HT$_{1A}$ receptor. Efficacy values (E_{max}) are the maximal stimulation achieved expressed as a percentage of the maximal 5-HT response. Results are arithmetic means ± S.E.M. of 3–4 experiments. For details of experimental conditions, see Section 2 and Section 2.3.

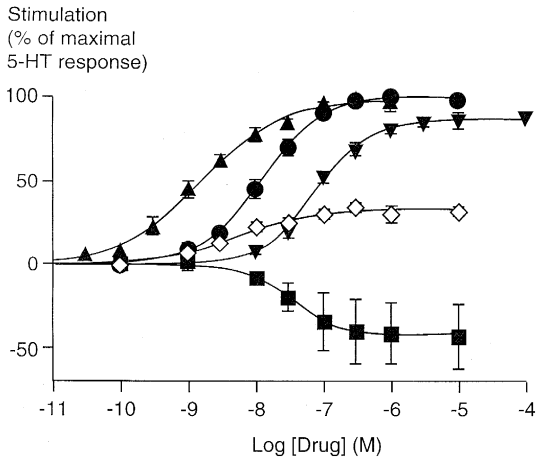

Fig. 2. Dose–response curves for 5-HT (●), 5-CT (▲), buspirone (▼) and NAN 190 (◇)-induced stimulation, and methiothepin (■)-induced inhibition, of [^{35}S]GTPγS binding at the cloned human 5-HT$_{1A}$ receptor stably expressed in HeLa cells. Each curve is a composite of 3–4 separate experiments ± S.E.M. Potency values are given as pD_2 and efficacy values are given as a percentage of the maximal response seen with 5-HT. The pD_2 value for 5-HT was 7.9. 5-CT and buspirone were full agonists with respect to 5-HT yielding efficacy values of 98% and 86%, respectively, and potency values of 8.8 and 7.1, respectively. NAN 190 was a partial agonist (pD_2 value 8.3, efficacy 32%) and methiothepin an inverse agonist (efficacy −43%). For details of experimental conditions, see Section 2 and Section 2.3.

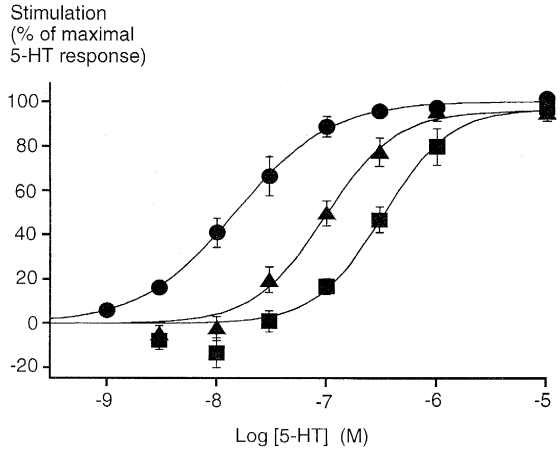

Fig. 3. Composite dose–response curves (means ± S.E.M., $n = 3$) for 5-HT-induced [^{35}S]GTPγS binding mediated by the cloned human 5-HT$_{1A}$ receptor stably expressed in HeLa cells in the absence (●) and presence of 30 nM (▲) and 100 nM (■) methiothepin. Methiothepin produced a dose-dependent parallel rightward shift of the 5-HT dose–response curve with no apparent reduction in E_{max} indicative of competitive antagonism. The apparent pA_2 value obtained using both concentrations of methiothepin was 8.4. For details of experimental conditions, see Section 2 and Section 2.3.

respectively (mean ± S.E.M., $n = 10$). 5-CT, 8-OH-DPAT, RU24969, buspirone, gepirone, mesulergine and sumatriptan were all full agonists with respect to 5-HT, whilst NAN 190 was a partial agonist and methiothepin an inverse agonist. Potency (pD_2) and efficacy (E_{max}) values are given in Table 2 and examples of dose–response curves are given in Fig. 2.

The [^{35}S]GTPγS binding stimulated in the presence of 10 μM 5-HT, 10 μM 8-OH-DPAT and 10 μM 5-HT plus 10 μM 8-OH-DPAT was 155 ± 15, 153 ± 17 and 154 ± 18 fmol/mg protein (mean ± S.E.M., $n = 3$), respectively.

Methiothepin (30 nM and 100 nM), produced a parallel rightward shift in the 5-HT dose–response curve with no apparent reduction in the E_{max} (Fig. 3), indicative of competitive antagonism and yielded an apparent pA_2 value of 8.4 ± 0.1 (mean ± S.E.M., $n = 3$).

3.3. Benextramine studies

Pre-treatment with the alkylating agent, benextramine (3, 6 and 10 μM) for 60 min resulted in an irreversible antagonism of 5-HT-induced [^{35}S]GTPγS binding to the cloned human 5-HT$_{1A}$ receptor, stably expressed in HeLa cells. At 3 μM benextramine this resulted in a rightward shift of the 5-HT dose–response curve. At higher concentrations (6 and 10 μM) the dose–response curve was shifted rightwards and the maximal 5-HT response depressed. Dose–response curves for this response with and without pre-treatment with benextramine (3, 6 and 10 μM) are given in Fig. 4a. A reduction in the receptor constitutive activity, detected as a lowering of the baseline activity following receptor blockade with benextramine, was ob-

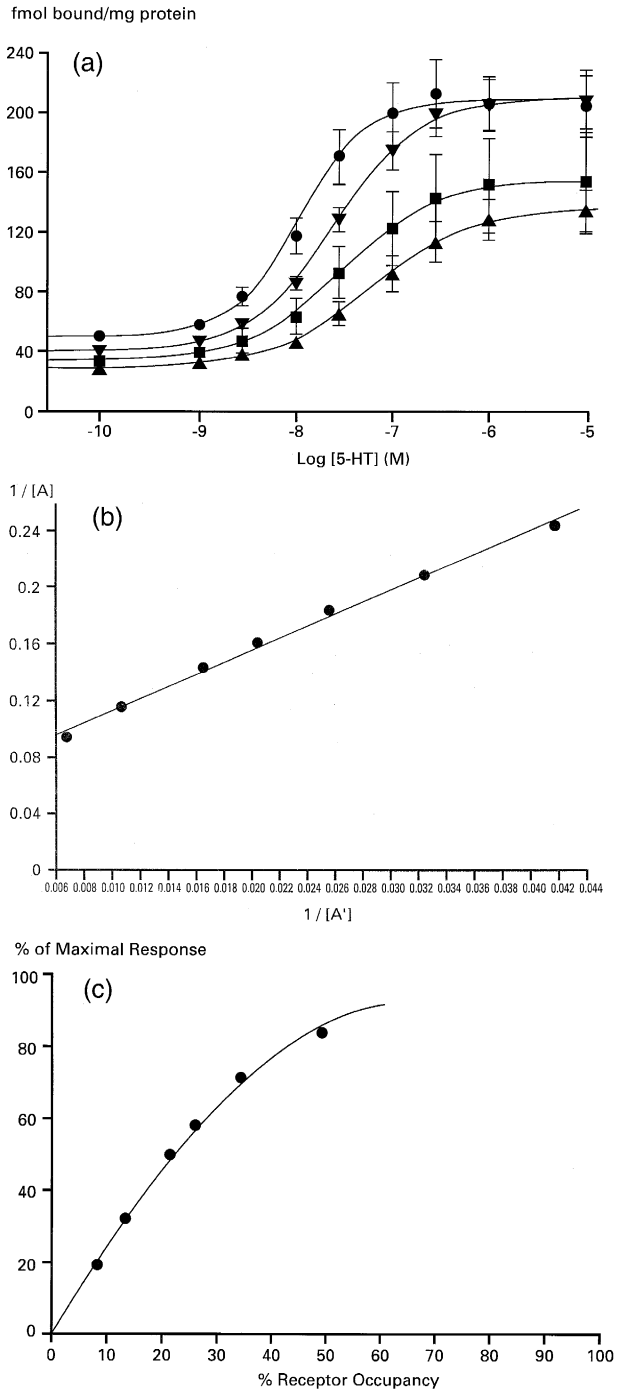

Fig. 4. Composite dose–response curves (means ± S.E.M., $n = 3$) for 5-HT-induced [^{35}S]GTPγS binding mediated by the cloned human 5-HT$_{1A}$ receptor stably expressed in HeLa cells before (●) and after pre-treatment with 3 μM (▼), 6 μM (■) and 10 μM (▲) benextramine (panel a). Stimulation of 5-HT-induced [^{35}S]GTPγS binding levels are expressed in fmol bound/mg protein. Pre-treatment with 3 μM benextramine resulted in a rightward shift of the 5-HT dose–response curve. At higher concentrations (6 μM and 10 μM) the dose–response curve was shifted rightwards and the maximal 5-HT response depressed. A comparison of equiactive doses of 5-HT with and without 10 μM benextramine (panel b) yielded a pK_A value of 7.3. A comparison of receptor occupancy versus response (panel c) indicated that a 22% receptor occupancy is required to elicit a half-maximal (50%) response in this cell line.

served in these studies and ranged from 50 ± 3 fmol [^{35}S]GTPγS bound/mg protein (mean ± S.E.M., $n = 3$) under basal conditions to 28 ± 2 fmol [^{35}S]GTPγS bound/mg protein (mean ± S.E.M., $n = 3$) following pre-treatment with 10 μM benextramine. A comparison of the reciprocal values of equiactive doses of 5-HT before and after pre-treatment with 10 μM benextramine is shown in Fig. 4b. These studies yielded a pK_A value for 5-HT of 7.3 ± 0.1 (mean ± S.E.M., $n = 3$). A comparison of receptor occupancy versus response indicates that, in the HA6 cell line, a $20 \pm 3\%$ (mean ± S.E.M., $n = 3$) receptor occupancy by 5-HT is required to elicit a half-maximal (50%) response (Fig. 4c).

4. Discussion

Characterisation of novel agonists requires an assessment of not only their affinities at the receptor of interest but also a measure of their relative efficacies and potencies. Such problems encountered with receptor agonist characterisation is no better illustrated than for 5-HT$_{1A}$ receptor ligands. For example the anxiolytic/antidepressant drugs buspirone and gepirone have been varyingly described as 5-HT$_{1A}$ receptor full agonists, partial agonists and antagonists. Such discrepant findings have lead, erroneously, to the suggestion of multiple 5-HT$_{1A}$ receptor subtypes (Dumuis et al., 1988) but are now thought to be due to the varying degrees of receptor reserve present in the model system being used, i.e., the receptor density (Hoyer and Boddeke, 1993), secondary messenger coupling efficiency (Gettys et al., 1994) and compound intrinsic activity. Variations in the degree of receptor reserve are encountered in systems using recombinant receptors stably expressed in cell lines (Boddeke et al., 1992) and, indeed, in native tissue. Hence, it is now well established that pre-synaptic 5-HT$_{1A}$ receptors in raphe regions occur with a relatively higher degree of receptor reserve than post-synaptic hippocampal 5-HT$_{1A}$ receptors (Yocca et al., 1992; Meller et al., 1990).

Cloned receptors stably expressed in 'foreign' cell lines, i.e., cell lines in which they are not normally expressed, are increasingly used as model systems for determining the affinities, potencies and relative efficacies of novel compounds. Such cell lines, consisting of essentially homogeneous receptor populations, usually expressed in higher densities than normally seen in native tissues, offer obvious advantages. A knowledge of the degree of receptor reserve in such systems, as, indeed, is the case with native tissue, allows for a more meaningful interpretation of the potency and efficacy measures of novel compounds.

In this present study we have taken advantage of the agonist-induced [^{35}S]GTPγS binding, to assess the receptor reserve, with respect to the endogenous ligand 5-HT, in a HeLa cell line stably transfected with the cloned human 5-HT$_{1A}$ receptor, the HA6 cell line. HeLa cell lines stably transfected with rat and human 5-HT$_{1A}$ receptors have previously been characterised (Fujiwara et al., 1993; Pauwels et al., 1993; Fargin et al., 1989). Additionally, however, the present study describes a convenient method of determining the receptor reserve in the HA6 cell line which could, theoretically, be applied to native tissue or further cell lines stably expressing this or other G-protein-coupled receptors.

Initial [^{3}H]5-HT radioligand binding saturation studies resulted in a composite curve best fit to a one site model and yielding a pK_{obs} value of 8.4 and a B_{max} value of 10 000 fmol/mg protein. The inability to detect the presence of high and low affinity state in the [^{3}H]5-HT saturation studies may be due to the limited assay resolution resulting from the restricted number of ligand concentrations used. The high density of 5-HT$_{1A}$ receptors expressed in this HA6 cell line, at least compared with that often seen in native tissue, has been reported previously (Fargin et al., 1989; Hoyer and Boddeke, 1993).

[^{3}H]5-HT and [^{3}H]8-OH-DPAT displacement studies were carried out on 20 standard compounds and the resulting pK_i values were in excellent agreement yielding a correlation coefficient $r = 0.99$. Both radioligands were also guanyl nucleotide sensitive (GTPγS > GTP > GDP) and ATP insensitive (data not given) indicating the labeling of high affinity 5-HT$_{1A}$ receptors at the concentrations of radioligand used.

Under control conditions 5-HT caused a dose-dependent increase in [^{35}S]GTPγS binding with a maximal level of 210 fmol/mg protein, compared to a basal level of 81 fmol/mg, and an EC_{50} value of 13 nM. This response is very probably 5-HT$_{1A}$ receptor mediated since the levels of [^{35}S]GTPγS bound with two full agonists (5-HT and 8-OH-DPAT) were not additive. 10 μM 5-HT, 10 μM 8-OH-DPAT and 10 μM 5-HT plus 10 μM 8-OH-DPAT yielded values of 155, 153 and 154 fmol/mg protein, respectively.

Pre-treatment of the cells, for 1 hour, with the alkylating agent benextramine (3 μM) resulted in a small rightward shift of the 5-HT dose–response curve, and at higher concentrations (6 and 10 μM), in a depression of the maximal response elicited by 5-HT. A comparison of equiactive doses of 5-HT before and after the benextramine pre-treatment and an analysis of the data by the double-reciprocal method of Furchgott yielded a pK_A value of 7.3. This discrepancy between the K_A and K_{obs} values indicates that the 5-HT$_{1A}$ receptor, in this cell line, is present in both high and low affinity states. Calculation of the fractional receptor occupancy for each concentration of agonist, revealed a non-linear relationship between receptor occupancy and response to 5-HT, indicating the presence of receptor reserve whereby a half-maximal (50%) response was achieved with a 20% receptor occupancy. This is in good agreement with work described by Boddeke et al. (1992) where the effects of compounds on intracellular calcium concentrations were investigated and

the presence of receptor reserve in the HA6 cell line demonstrated.

The HA6 cell line was then used to investigate a series of compounds, 5-HT, 5-CT, 8-OH-DPAT, RU24969, sumatriptan, buspirone, gepirone, mesulergine, NAN 190 and methiothepin, in terms of their potencies and relative efficacies, using the agonist-induced [^{35}S]GTPγS binding assay. The equally efficacious agonists yielded the following typical 5-HT_{1A} receptor pharmacological profile: 5-CT > 8-OH-DPAT > 5-HT ≥ RU24969 > buspirone > gepirone > mesulergine ≥ sumatriptan. The pD_2 values obtained with these compounds was in excellent agreement with their corresponding pK_i values obtained in both the [^{3}H]5-HT and [^{3}H]8-OH-DPAT radioligand binding displacement studies. The presence of receptor reserve would normally be expected to result in higher potency values compared with binding affinities, however, the use of relatively low concentrations of an agonist radioligand in this study results in a measure of binding to the high affinity recognition site only and probably accounts for the apparent anomalous agreement between the potencies and binding affinities seen with these compounds.

As expected, the agonists 5-CT, 8-OH-DPAT and RU24969 were all fully efficacious compared with 5-HT. The anxiolytic compounds buspirone and gepirone have been shown to act as full and partial agonists in differing assay systems (Hoyer and Boddeke, 1993) and therefore it is not surprising that in this study using a cell line known to display the presence of receptor reserve with respect to 5-HT, both compounds display full agonism. This is in contrast with the studies by Raymond et al., 1992, in which buspirone was shown to be devoid of intrinsic activity (inhibiting forskolin-stimulated adenylyl cyclase) at the cloned human 5-HT_{1A} receptor, stably transfected in CHO cells, whilst 5-CT and 8-OH-DPAT were as efficacious as 5-HT. Such discrepancies further illustrate the usefulness of characterising stably transfected cell lines to allow for a more meaningful interpretation of experimental data. The weak partial agonism seen with NAN 190 was also not unexpected as this compound has been shown to act as an antagonist in post-synaptic receptor models, where the receptor reserve is low, and as an agonist in pre-synaptic models where the receptor reserve is high (Hjorth and Sharp, 1990; Rydelek-Fitzgerald et al., 1990). More interestingly, however, were the results obtained with the non-selective 5-HT_1/5-HT_2 receptor agent methiothepin which has previously been classed as a non-selective 5-HT_{1A} receptor antagonist. In the present study methiothepin clearly displayed inverse agonism suggesting an ability to inhibit the basal activity of the receptor probably by actively promoting the dissociation of the receptor from the G protein. This effect of methiothepin was further investigated following pre-treatment of the cells with benextramine. This largely abolished the response but this effect was probably due to the anticipated lowering of the receptor constitutive activity, as a direct consequence of the benextramine treatment, and hence an inability to detect inverse agonism (data not shown). It is possible that this apparent inverse agonism may be due to an antagonism of endogenous agonist. This, however, would also seem unlikely as the assay is carried out using washed membrane preparations. Indeed methiothepin, has also been shown to be an inverse agonist at cloned human 5-HT_{1D_α} and 5-HT_{1D_β} receptors stably transfected in CHO cells where neither the potency nor the magnitude of the inhibition of [^{35}S]GTPγS binding was significantly altered by repeated membrane washing steps (Thomas et al., 1995).

When used to antagonise 5-HT in the agonist-induced [^{35}S]GTPγS binding assay methiothepin yielded an apparent pA_2 value of 8.4 compared with a pK_i value of 7.8 obtained from radioligand binding studies. Normally one would expect excellent agreement between the antagonist affinity estimates obtained from binding and functional studies. One possible explanation for this is the presence of Na^+ ions in the agonist-induced [^{35}S]GTPγS binding assay which may result in a destabilisation of the precoupled receptor. Costa et al. (1992) have demonstrated a similar phenomenon for the opioid receptor endogenously expressed in NG108-15 cells. In this system ICI 174, a compound which displayed inverse agonism, yielded pA_2 values of 7.43 and 6.78 when antagonising the full opioid agonist [D-Ala^2,D-Leu^5]enkephalin (DADLE) in the presence and absence of Na^+ ions, respectively. An alternative explanation is the expected effect a compound displaying inverse agonism would have on the balance of high/low affinity states of G-protein-coupled receptors. This effect, which is opposite to that seen with an agonist and which would theoretically not occur with a 'silent' antagonist, would result in the increased formation of the low affinity state of the receptor which is favoured by the inverse agonist (Leff, 1995).

The phenomenon of inverse agonism is well established for the γ-aminobutyric acid type A ($GABA_A$) receptor, where β-carbolines elicit effects opposite to those seen with benzodiazepines, e.g., reduce the affinity of the receptor for GABA leading to seizure inducing and anxiogenic properties. Compounds displaying inverse agonism at G-protein-coupled receptors (Milligan et al., 1995) could potentially be exploited in the treatment of diseases which are due to constitutively active mutated G protein-coupled receptors, e.g., familial male precocious puberty (Shenker et al., 1993) and hyperfunctioning thyroid adenomas (Parma et al., 1993). This study demonstrates the power of the agonist-induced [^{35}S]GTPγS assay in providing an efficient means of identifying such compounds.

In conclusion, here we describe a functional assay, the agonist-induced [^{35}S]GTPγS binding in a HeLa cell line stably expressing cloned human 5-HT_{1A} receptors (HA6). This assay has been used to efficiently characterise the cell line in terms of receptor reserve with respect to 5-HT and the nature of the receptor affinity states. It has also been

shown to have sufficient resolution to identify full and partial agonists, silent antagonists, and perhaps more importantly, compounds displaying inverse agonism. Although such cell lines do not necessarily mirror situations seen in native tissue such information regarding receptor reserve allows for a more meaningful interpretation of the nature of novel compound and hence an ability to more accurately predict their effects at central neurons.

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