British Journal of Pharmacology (1996) 117, 1119-1126

Characterization of a 5-HT_{1B} receptor on CHO cells: functional responses in the absence of radioligand binding

[1]Heather Giles, S.J. Lansdell, M.-L. Bolofo, H.L. Wilson & G.R. Martin

Receptor Pharmacology Group, Biology Division, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 Chinese hamster ovary (CHO) cells have been reported to be devoid of 5-HT receptors and have frequently been used as hosts for the expression of cloned 5-HT receptors. Unexpectedly, 5-HT was found to induce profound inhibition of forskolin-stimulated cyclic AMP production in these cells and the aim of this study was to classify the 5-HT receptor involved.

2 In CHO(dhfr-) cells 5-HT was a potent agonist and caused 80-100% inhibition of forskolin stimulated cyclic AMP production. A study using several 5-HT_1 receptor agonists revealed the following potencies ($p[A_{50}]$): RU24969 (9.09 ± 0.17) > 5-carboxamidotryptamine (8.86 ± 0.20) > 5-HT (8.07 ± 0.05) > CP-93,129 (7.74 ± 0.10) > sumatriptan (5.93 ± 0.04). All five agonists achieved a similar maximum effect. Irreversible receptor alkylation studies yielded a pK_A estimate of 7.04 ± 0.34 for 5-HT.

3 The 5-$HT_{1A/1B}$ antagonist, (±)-cyanopindolol (4-100 nM), caused parallel rightward shifts of the 5-HT concentration-effect curve with no change in asymptote. Schild analysis yielded a pK_B estimate of 8.69 ± 0.09 (Schild slope 1.13 ± 0.10). (±)-Cyanopindolol actually behaved as a partial agonist with an intrinsic activity of 0.2-0.5 and a $p[A_{50}]$ of 8.55.

4 5-HT (0.01-10 μM) also elicited a concentration-dependent increase in intracellular $[Ca^{2+}]$ in CHO(dhfr-) cells thus demonstrating that dual coupling is not a phenomenon restricted to systems in which there is overexpression of transfected receptors.

5 This agonist and antagonist profile is consistent with the presence of a 5-HT_{1B} receptor. 8-OH-DPAT (1 μM) and renzapride (3 μM) were without effect on forskolin-stimulated cyclic AMP production and ketanserin (0.3 μM) did not antagonize the inhibition produced by 5-HT, thus excluding the involvement of 5-HT_{1A}, 5-HT_4, and 5-HT_2 receptors.

6 The possibility that expression of a 5-HT_{1B} receptor was associated with the dhfr- mutation was excluded since RU24969, 5-HT and CP-93,129 were also potent agonists in unmutated, CHO-K1 cells: $p[A_{50}]$ 9.03 ± 0.03, 8.34 ± 0.05, 7.69 ± 0.07 respectively, and (±)-cyanopindolol (0.1 μM) shifted the 5-HT curve to the right and yielded a pA_2 estimate of 8.70 ± 0.06.

7 Little or no specific binding of [^{3}H]-5-HT (0.1-200 nM) or of the high affinity ligand [^{125}I]-iodocyanopindolol (0.01-3 nM) to CHO(dhfr-) cell membranes could be detected. 5-HT also failed to elicit any increase in the binding of [^{35}S]-GTPγS to CHO membranes.

8 In conclusion, cultured CHO cells express 5-HT_{1B} receptors which are negatively coupled to adenylyl cyclase and positively coupled to increases in intracellular calcium. The absence of radioligand binding was unexpected in view of the high potency of 5-HT and the partial agonist activity of the normally 'silent' competitive antagonist, (±)-cyanopindolol. This implies very efficient receptor-effector coupling of a low density of 5-HT_{1B} receptors. Clearly, the absence of detectable radioligand binding cannot be assumed to mean the absence of receptors capable of eliciting a significant functional response.

Keywords: CHO cells; 5-HT_{1B} receptor; receptor classification; cyclic AMP; GTPγS; binding

Introduction

The use of cloned receptors stably expressed in cell lines provides an increasingly important tool in receptor pharmacology. Many human receptors which have previously been inaccessible can now be studied, albeit not in their native cell, provided the gene encoding for the receptor has been isolated, cloned and expressed.

Chinese hamster ovary (CHO) cells are frequently used as the host for cloned receptors particularly a mutant CHO cell line (CHO(dhfr-)) which lacks the dihydrofolate reductase gene (Urlab & Chasin, 1980) and therefore cannot replicate in the absence of exogenous thymidine and hypoxanthine. Genes for both the receptor and the dhfr gene are transfected into CHO(dhfr-) cells, which are then grown in the absence of thymidine and hypoxanthine in order to select only the cells which are expressing the transfected genes.

An important consideration when choosing a cell line for the expression of a cloned receptor is the absence of natively expressed receptors. Untransfected CHO cells are frequently reported to be devoid of 5-HT receptors and have been used for the expression of recombinant 5-HT_1 receptor genes (Raymond, 1991; Newman-Tancredi *et al.*, 1992; Veldman & Bienkowski, 1992; Thomas *et al.*, 1995). In agreement with others, our preliminary experiments failed to detect any significant [^{3}H]-5-HT binding to CHO(dhfr-) cell membranes. Unexpectedly, in the present study 5-HT elicited profound and potent inhibition of forskolin-stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) production. The aim of this study was to identify the receptor involved in this response and to try and understand how such a potent response could be elicited in the absence of any detectable radioligand binding.

[1] Author for correspondence.

Methods

Cell culture

CHO(dhfr-) and CHO-K1 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM, glucose 4.5 g l^{-1}) supplemented with 10% (v/v) dialysed foetal calf serum. L-glutamine (2 mM), non-essential amino acids (5 ml 500 ml^{-1}), hypoxanthine (100 μM) and thymidine (16 μM) (HT solution), gentamicin (20 μg ml^{-1}), penicillin (100 u ml^{-1}) streptomycin (100 μg ml^{-1}) and amphotericin (0.25 μg ml^{-1}) (antibiotic-antimycotic solution). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

Preparation of cell membranes

CHO (dhfr-) cells were grown to confluence in 175 cm^2 flasks (Falcon). Cells were washed with preparation buffer: 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA and 6 mM $MgCl_2$, detached with a cell scraper into 10 ml preparation buffer and homogenized (Ystral homogeniser, 10s at 4°C). The homogenate was centrifuged (170,000 g) for 60 min, at 4°C (Beckman ultracentrifuge) and the resulting pellet was resuspended in incubation buffer: 50 mM Tris HCl pH 7.4, containing 5 mM $CaCl_2$, 0.1% (v/v) ascorbic acid to prevent indolamine oxidation and 10 μM pargyline, a monoamine-oxidase inhibitor. Protein determination was carried out with a Bio Rad kit with bovine serum albumin as a standard: the final protein yield was 0.7–1.0 mg per 175 cm^2 flask of cells. Membranes were stored at −70°C at a concentration of approximately 1 mg protein ml^{-1}, and were generally used within 2 weeks of preparation.

Radioligand saturation binding assays

Binding assays were performed in a total volume of 1 ml. All components of the assay were diluted in incubation buffer and all results obtained as duplicates. Tubes contained increasing concentrations of [^{3}H]-5-HT (0.1–200 nM) or [^{125}I]-iodocyanopindolol (0.01–3 nM) in the absence or presence of an excess of unlabelled 5-HT (100 μM) to define non-specific binding. The incubation was started by the addition of 30 μg membrane protein. After 30 min at 27°C the reaction was terminated using a Brandel harvester by addition of excess Tris buffer pH 7.4 (4°C) and rapid vacuum filtration through Whatman GF/C filters. Radioactivity was measured by liquid scintillation counting (Beckman LS5000TD scintillation counter) using Optiphase HiSafe scintillant (Wallac).

[^{35}S]-GTPγS binding assay

Assays were performed in a total volume of 100 μl, using the following assay buffer: 10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, 150 mM NaCl, 10 μM guanosine-5'-di-phosphate (GDP), 1 mM dithiothreitol. Data were obtained in triplicate.

Membranes were pre-incubated in assay buffer for 10 min. Binding of 0.2 nM [^{35}S]-GTPγS was determined in the presence of increasing concentrations of 5-HT (1 nM–100 μM) and the incubation was started by the addition of 3 μg of membrane protein to each assay tube. Incubation conditions, harvesting and measurement of radioactivity were as described above.

Cyclic AMP assay

Cells were cultured in 12-well plates (Falcon) until confluent. The medium was removed and, following a wash with PBS (phosphate buffered saline), replaced with modified DMEM containing 20 mM HEPES, 100 μM IBMX (iso-butyl-methylxanthine) to inhibit phosphodiesterase and 10 μM pargyline. The cells were equilibrated for at least 20 min in a humidified 37°C incubator prior to addition of antagonist or vehicle which was incubated for a further 45 min. For irreversible alkylation studies, benextramine tetrahydrochloride (BHC, 3–10 μM) was incubated for 30 min followed by two washes with PBS prior to addition of fresh medium for a further 45 min. The appropriate concentration of agonist was then added to each well, immediately followed by 1 μM forskolin. The plates were incubated for exactly 10 min at 37°C and the reaction was then stopped by aspiration of the medium and addition of 400 μl ice cold ethanol. Cyclic AMP was extracted into the ethanol and after 2 h at room temperature, 50 μl ethanol from each well was transferred into 96-well sample plates (Wallac) and evaporated to dryness. Cyclic AMP determination was carried out by [^{125}I]-cyclic AMP scintillation proximity assay (SPA). A single agonist concentration-effect curve was performed in each plate in addition to measurement of basal and forskolin-stimulated cyclic AMP levels. Duplicate concentration-effect curves were generated on two different plates. Basal levels of cyclic AMP accumulation were 0.5–1 pmol/well. Absolute values for forskolin-stimulated cyclic AMP accumulation varied between experiments (7–30 pmol/well) but variability within an experiment was generally less than 10%.

Intracellular calcium measurements

CHO(dhfr-) cells were grown to confluence in 175 cm^2 flasks. Cells were washed with PBS, detached using trypsin (2.5%) in versene and resuspended in 20 ml DMEM containing 5 μM FURA-2/AM, 0.02% (v/v) pluronic and 250 μM sulphinpyrazone. The cell suspension was incubated at 37°C for 45 min then centrifuged at 760 g for 10 min (Jouan CR-312 centrifuge). The cell pellet was resuspended in 20 ml of assay medium (DMEM plus 250 μM sulphinpyrazone). After 15 min equilibration at room temperature the cells were centrifuged at 760 g for a further 10 min and resuspended in assay medium (5–10 ml) to yield a final cell count of $2-5\times10^6$ cells ml^{-1}. Aliquots of the cell suspension (0.5 ml) were placed in fluorescence cuvettes with a stirrer and 5-HT-induced changes in fluorescence were measured in a spectrofluorophotometer (excitation $\lambda=340$ nm, emission $\lambda=490$ nm: Shimadzu RF-5001PC). Digitonin (1 mM) and manganese chloride (10 mM) were used to define maximum and minimum fluorescence respectively.

Analysis of cyclic AMP data

Agonist curve fitting: Concentration-effect curves were fitted to a logistic function of the form:

$$E = \beta + \frac{(\alpha - \beta)}{1 + \exp(-2.303\ m(\log_{10}[A] - \log_{10}[A_{50}]))} \quad \text{Eq 1}$$

in which E, β, α, m and $[A_{50}]$ are effect, minimum asymptote, maximum asymptote slope coefficient and mid-point curve location parameters, respectively. Arithmetic means of $p[A_{50}]$ ($-\log_{10}[A_{50}]$) estimates together with the standard error are quoted in the text.

Analysis of antagonism: One way analysis of variance tested for antagonist effects on computed estimates of α and m. If the antagonist did not significantly modify these parameters then computed $p[A_{50}]$ values were fitted to the following form of the Schild equation (Black *et al.*, 1985):

$$\log_{10}[A_{50}] = \log_{10}[A_{50}^c] + \log_{10}(1 + [B]^n/K_B) \quad \text{Eq 2}$$

Where $[A_{50}^c]$ is the control $[A_{50}]$ value, [B] is the concentration of antagonist, K_B is the antagonist dissociation equilibrium constant and n is the order of reaction with the receptor. If n was not significantly different from unity, consistent with simple competition, it was constrained to this value in order to obtain an estimate of K_B. The dissociation constant was actually estimated as pK_B ($-\log_{10}K_B$). In some experiments only

one concentration of antagonist was used and in these cases the resulting concentration ratio was used to calculate a pA_2, using the Schild equation:

$$\log(r - 1) = \log[B] + pA_2 \quad \text{Eq 3}$$

where r is the concentration ratio: $[A_{50}]/[A_{50}^c]$

Receptor inactivation studies: Estimates of agonist affinity and efficacy were derived by direct operational model-fitting methods (Black & Leff, 1983; Leff *et al.*, 1990). Agonist concentration-effect curve data, obtained before and after fractional receptor alkylation, were fitted directly to:

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad \text{Eq 4}$$

in which K_A is the dissociation equilibrium constant, τ is agonist efficacy, E_m is the maximum possible effect in the receptor system and n defines the slope coefficient of the occupancy-effect relation. Error estimates were obtained as described in detail previously (Leff *et al.*, 1990).

Materials

Dulbecco's modified Eagle medium (DMEM), L-glutamine, Cellect 50X HT solution (5000 μM hypoxanthine, 800 μM thymidine) and trypsin were purchased from ICN Flow (High Wycombe), dialysed foetal calf serum from Advanced Protein Products (Brierley Hill), phosphate buffered saline w/o calcium and magnesium, versene 1:1000, MEM non-essential amino-acids X100 and antibiotic-antimycotic solution from Gibco Laboratories (Paisley).

Gentamicin sulphate, DMEM with HEPES modification, isobutyl-methyl-xanthine (IBMX), forskolin, 5-HT creatinine sulphate, (±)-8-hydroxy-2-(di-n-propyl amino) tetralin (8-OH-DPAT), pargyline HCl, dithiothreitol (DTT), (±)-sulphinpyrazone and digitonin were purchased from Sigma (Poole). Guanosine-5'-diphosphate (GDP) was from Boehringer Mannheim (GmbH), benextramine $(HCl)_4$ from RBI (St Albans), pluronic + F−127 protein grade detergent was purchased from Calbiochem (Nottingham) and FURA-2/AM was from Cambridge Bioscience (Cambridge). Albumin standard was purchased from Pierce (Rockford, Illnois, U.S.A.), and the Bio-Rad protein assay was from Bio-Rad laboratories (Munich, Germany).

5-Carboxamidotryptamine hydrochloride (5-CT) and sumatriptan (3-[2-dimethylamino]ethyl-n-methyl-1H-indole-5 methane sulphonamide hydrochloride) were synthesized by Dr A.D. Robertson, Medicinal Chemistry Department, Wellcome Research Laboratories, Kent. [^{3}H]-5-HT (25 Ci mmol^{-1}) and [^{35}S]-GTPγS (1390 Ci mmol^{-1}) were purchased from Du Pont-NEN (Stevenage), high specific activity [^{3}H]-5-HT (99 Ci mmol^{-1}), -(3-)[^{125}I]-iodocyanopindolol (2,000 Ci mmol^{-1}) and cyclic AMP [^{125}I] scintillation proximity assay (SPA, dual range) were purchased from Amersham International (Bucks).

We are grateful to the following companies for gifts of compound: RU24969 succinate (5-methoxy-3(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole) (Roussel Uclaf, Romainville, France), ketanserin tartrate (Janssen Pharmaceutica, Beerse, Belgium), (±)-cyanopindolol fumarate (Sandoz Ltd, Basle, Switzerland), CP-93,129 (5-hydroxy-3(4-1,2,5,6-tetrahydropyridyl)-4-azaindole) (Pfizer, Groton, CT, U.S.A.), renzapride ((±)-endo-4-amino-N-(azabicyclo[3.3.1.]non-4-yl)-5-chloro-2-methoxybenzamine) (SmithKline Beecham, Harlow).

Results

Inhibition of forskolin-stimulated cyclic AMP accumulation by 5-HT$_1$ receptor agonists in CHO(dhfr-) cells

5-HT potently and concentration-dependently inhibited forskolin-stimulated cyclic AMP accumulation - $p[A_{50}]$ 8.07±0.05 (n=5); the maximum response elicited being 80–100% inhibition (Figures 1 and 2). Figure 1 also shows the effect of the following 5-HT$_1$ receptor agonists on inhibition of forskolin (1 μM)-stimulated cyclic AMP accumulations: 5-CT, RU24969, a 5-HT$_{1A/B}$ receptor agonist, CP-93,129, a highly selective ligand for 5-HT$_{1B}$ receptors, and sumatriptan. All compounds achieved a similar maximum effect of 80–100% inhibition and demonstrated the following potency order: RU24969 > 5-CT > 5-HT > CP-93,129 > sumatriptan (n=4–6). Potency and potency ratios are shown in Table 1.

8-OH-DPAT (1 μM) and renzapride (3 μM) were without effect on forskolin-stimulated cyclic AMP production and ketanserin (0.3 μM) did not antagonize the inhibition produced by 5-HT, thus excluding the involvement of 5-HT$_{1A}$, 5-HT$_4$ or 5-HT$_{2A}$ receptors.

Estimation of agonist affinity of 5-HT in CHO(dhfr-) cells

Estimates of the affinity (pK_A) of 5-HT were obtained in the cyclic AMP assay following partial receptor occlusion with BHC (3–10 μM), which elicited a rightward-shift and, at higher concentrations, a reduction in the upper asymptote of the 5-HT concentration-effect curve (Figure 2). The operational model of agonism (Eq 4) was fitted simultaneously to concentration-effect curve data from four separate experiments, which yielded the following average parameter estimates: pK_A 7.04±0.34, E_m 85.4±6.5, n 1.73±0.61 (12 d.f.). The average efficacy (τ) estimate for the control 5-HT curves was 9.27±0.36 (n=4). BHC alone had no significant effect on basal or forskolin-stimulated cyclic AMP levels.

Antagonism by (±)-cyanopindolol of the 5-HT-mediated inhibition of cyclic AMP in CHO(dhfr-) cells

Increasing concentrations of the 5-HT$_{1A/B}$ receptor antagonist (±)-cyanopindolol (4–100 nM) caused a parallel rightward

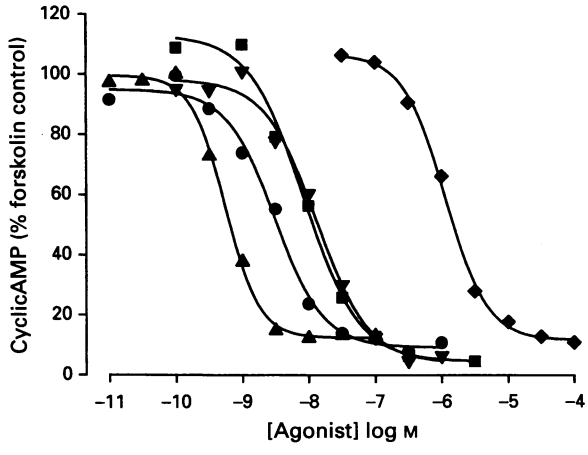

Figure 1 5-HT$_1$ receptor agonist concentration-effect curves for 5-HT (■), 5-CT (●), RU24969 (▲), CP-93,129 (▼), and sumatriptan (◆) in CHO(dhfr-) cells. Cells were stimulated with 1 μM forskolin and responses are expressed as % inhibition of the forskolin-induced increase in cyclic AMP. Data points are the mean of duplicates and the line through each data set is the result of fitting the individual data points to Eq 1.

shift of the 5-HT concentration-effect curve with no change in upper asymptote (Figure 3a). Schild analysis yielded a pK_B estimate of 8.69 ± 0.09 (13 d.f.) with a Schild slope which was not significantly different from unity (1.13 ± 0.10) (Figure 3b). (±)-Cyanopindolol actually behaved as a partial agonist (Figure 3c), causing a maximum of 20–50% inhibition of forskolin-stimulated cyclic AMP (n = 11), with a p[A_{50}] of 8.55 (n = 2).

The effect of 5-HT on intracellular [Ca^{2+}] in CHO(dhfr-) cells

5-HT (0.01 – 10 μM) caused a saturable, concentration-dependent increase in intracellular calcium concentration as measured by change in fluorescence in Fura-2 loaded cells. A maximum increase in fluorescence of 18.5 ± 1.6% of the response following cell lysis with digitonin (n = 4) was achieved at 1 μM 5-HT (Figure 4). Similar results were obtained with CP-93,129 (0.01 – 3 μM).

Pharmacological profile in CHO-K1 cells

CHO-K1 cells do not have the dhfr- mutation. 5-HT$_1$ receptor agonists potently inhibited forskolin-stimulated cyclic AMP accumulation in these cells: RU24969 > 5-HT > CP-93,129 (n = 3), (Figure 5a). Absolute potencies and potency ratios are displayed in Table 1. (±)-cyanopindolol (0.1 μM) caused a parallel rightward-shift of the 5-HT concentration-effect curve (Figure 5b) and yielded a pA_2 estimate of 8.70 ± 0.06 (n = 3).

Binding of high specific activity ligands to CHO(dhfr-) membranes

There was no significant difference between total and non-specific binding of [^{3}H]-5-HT, 25 Ci mmol^{-1} (0.1 – 200 nM) to CHO(dhfr-) cell membranes. The binding of the higher affinity, higher specific activity ligand, [^{125}I]-cyanopindolol (0.01 – 3 nM) was therefore examined together with high specific activity [^{3}H]-5-HT, 99 Ci mmol^{-1}. Examinations of the lower portion of the 5-HT curve revealed that, between 0.1 – 4 nM 5-HT, total binding was consistently greater than non-specific binding, suggesting the presence of a very small amount of specific binding (Figure 6). The [^{125}I]-iodocyanopindolol data showed a similar pattern.

The effect of 5-HT on [^{35}S]-GTPγS binding to CHO(dhfr-) membranes

5-HT (1nM – 10 μM) had no effect on the binding of 0.1 nM [^{35}S]-GTPγS to CHO(dhfr-) membranes (n = 3). In parallel experiments in CHO(dhfr-) cells transfected with the 5-HT$_{1A}$ receptor, 5-HT and the selective 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, elicited a concentration-dependent, saturable increase in [^{35}S]-GTPγS binding.

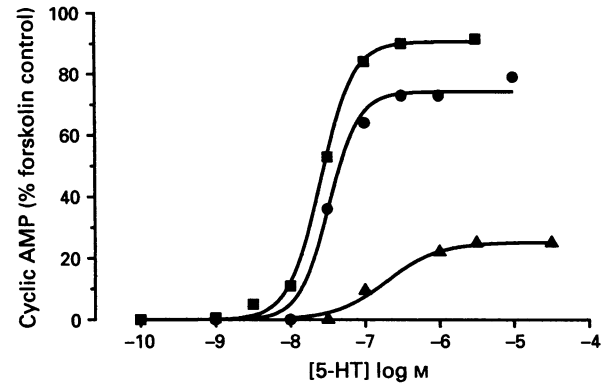

Figure 2 The effect of irreversible receptor alkylation by BHC, zero (■), 3 μM (●) and 10 μM (▲), on 5-HT-mediated inhibition of forskolin-stimulated cyclic AMP levels in CHO(dhfr-) cells. Representative data from a single day are shown. Data points are the mean of duplicates and the line through each data set is the result of fitting the individual data points to Eq 1.

Discussion

This paper provides strong evidence for the presence of a 5-HT$_{1B}$ receptor on CHO cells. 5-HT potently inhibited forskolin-stimulated cyclic AMP accumulation, in accordance with previous data demonstrating that activation of endogenous and transfected 5-HT$_{1B}$ receptors causes inhibition of adenylyl cyclase (Bouhelal *et al.*, 1988; Murphy & Bylund, 1989; Adham *et al.*, 1992; Shoeffter *et al.*, 1994). All the ligands tested behaved as full agonists and had potency ratios consistent with those previously reported at 5-HT$_{1B}$ receptors both for inhibition of adenylyl cyclase (Schoeffter & Hoyer, 1989; Schoeffter *et al.*, 1994), and for contraction of rat caudal artery (Craig & Martin, 1993). In particular, the highly selective 5-HT$_{1B}$ ligand, CP-93,129 (Macor *et al.*, 1990) was an agonist in CHO cells. 8-OH DPAT, a selective 5-HT$_{1A}$ receptor agonist, was without effect, suggesting that the effects of RU24969 and those of (±)-cyanopindolol, both of which have affinity for 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors, were due to action at the 5-HT$_{1B}$ receptor. (±)-Cyanopindolol behaved as a competitive antagonist of responses to 5-HT and the pK_B estimate of 8.69 was comparable to previous estimates at this receptor (Schoeffter &

Table 1 Potency estimates for inhibition of forskolin-stimulated cyclic AMP by 5-HT$_1$ agonists

Agonist	*CHO (dhfr-)* p[A_{50}] EMR	*CHO-K1* p[A_{50}] EMR	*Rat caudal artery** p[A_{50}] EMR
RU24969	9.09 ± 0.17 *0.1*	9.03 ± 0.03 *0.2*	8.72 *0.1*
5-CT	8.86 ± 0.20 *0.2*	–	8.14 *0.3*
5-HT	8.13 ± 0.07 *1*	8.34 ± 0.05 *1*	7.65 *1*
CP-93,129	7.74 ± 0.10 *2.5*	7.69 ± 0.07 *4.5*	7.37 *1.9*
Sumatriptan	5.93 ± 0.04 *158.5*	–	5.64 *102.3*

p[A_{50}] values ± s.e.mean are the averages of 4 – 6 estimates.
Values in italics are the equi-effective molar concentration ratios (EMR) relative to 5-HT
*Data from Craig & Martin, 1993

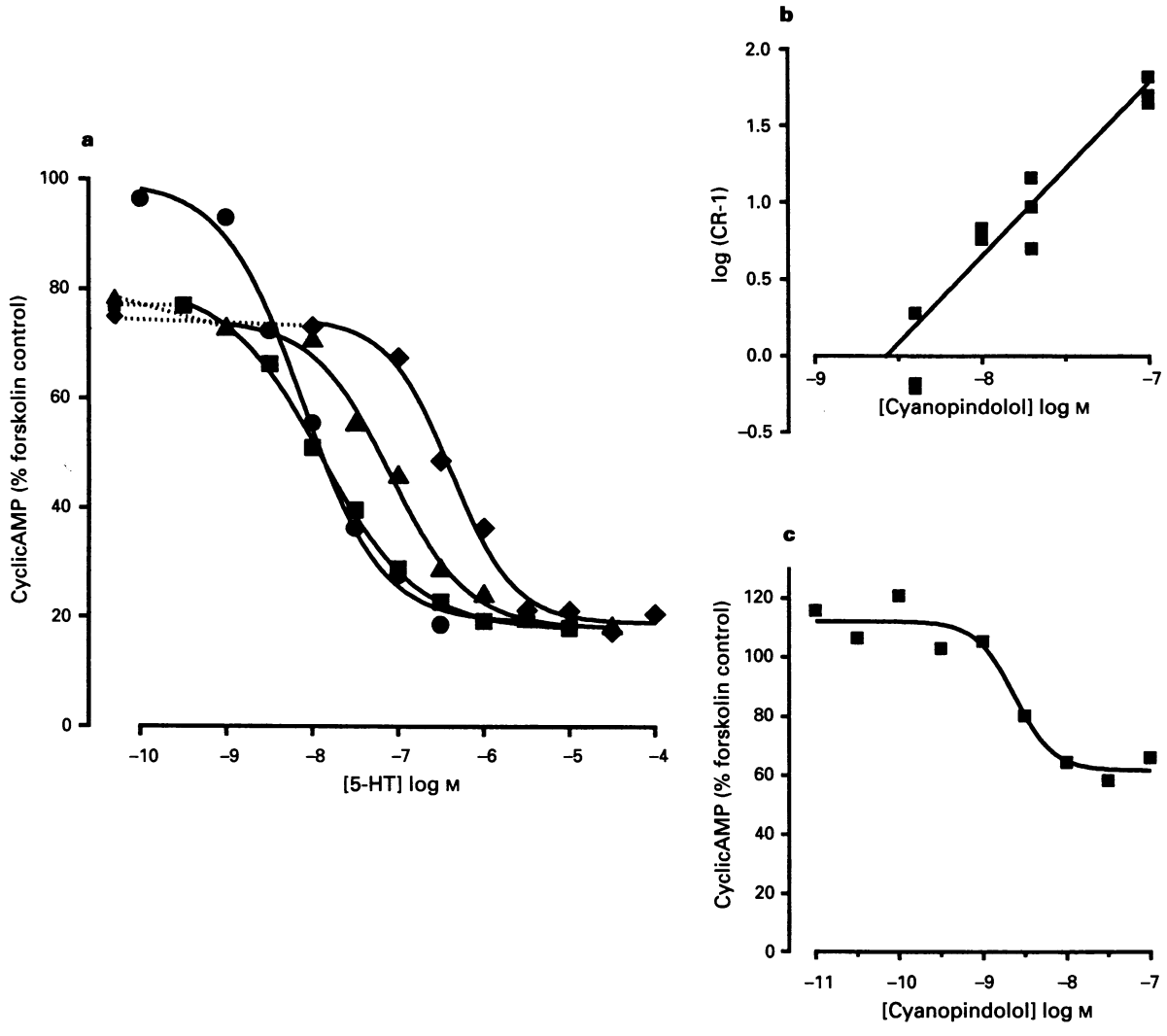

Figure 3 (a) Antagonism by (±)-cyanopindolol: zero (●), 4 nM (■), 20 nM (▲), and 100 nM (◆) of 5-HT-mediated inhibition of forskolin-stimulated cyclic AMP accumulation in CHO(dhfr-) cells. Representative data from a single day are shown. Data points are the means of duplicates and the line through each data set is the result of fitting the individual data points to Eq 1. Dotted lines connect the curve to the effect of (±)-cyanopindolol alone. (b) Schild plot: antagonism of 5-HT by (±)-cyanopindolol. The line through the data was calculated as described in the Methods section; $pK_B = 8.69 \pm 0.09$; slope $= 1.13 \pm 0.03$. (c) Representative (±)-cyanopindolol concentration-effect curve.

Hoyer, 1989; Craig & Martin, 1993). (±)-Cyanopindolol alone behaved as a partial agonist, p[A_{50}] 8.55, and caused 20–50% inhibition of forskolin-stimulated cyclic AMP accumulation. The similarity between p[A_{50}] and pK_B estimates implies that the agonism was 5-HT$_{1B}$ receptor-mediated, consistent with results in a variety of other functional 5-HT$_{1B}$ receptor assays (Engel *et al.*, 1986; Maura *et al.*, 1987; Murphy & Bylund, 1989; Schoeffter & Hoyer, 1989; Schoeffter *et al.*, 1994).

The susceptibility of CHO cell 5-HT$_{1B}$ receptors to irreversible alkylation by BHC enabled estimation of the affinity (pK_A) of 5-HT. The estimate of 7.04 is consistent with the reported affinity of 5-HT for the low affinity binding site in radioligand binding studies: 7.17–7.66 (Voigt *et al.*, 1992; Adham *et al.*, 1993). Recently Adham *et al.* (1993) have shown that EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) also alkylates the cloned rat 5-HT$_{1B}$ receptor, although high concentrations were found to be toxic.

Interestingly, 5-HT and the selective 5-HT$_{1B}$ receptor agonist, CP-93,129, also elicited concentration-dependent increases in intracellular free calcium, indicating that the 5-HT$_{1B}$ receptor may couple to two different G-proteins. Decreases in cyclic AMP are generally mediated through G_i and increases in calcium are often through G_q. Further studies are necessary to prove that the two effects are through different G-proteins in this cell, but if this is the case then the results demonstrate that the phenomenon of dual coupling is not just an anomaly observed in transfected cell lines with an abnormally high receptor density. It certainly seems likely that the smooth muscle contraction elicited by activation of 5-HT$_{1B}$ receptors in rat caudal artery (Craig & Martin, 1993) is due to increases in intracellular calcium, and not to inhibition of cyclic AMP. However, it may be that both mediators are being produced, since there is evidence that activation of the 5-HT$_{1D}$-like receptor in dog saphenous vein (possibly the mammalian species homologue of the rodent vascular 5-HT$_{1B}$ receptor) causes both contraction of smooth muscle and inhibition of adenylyl cyclase (Sumner *et al.*, 1992). Dual coupling, or at least the activation of several different transduction pathways by a single receptor may, therefore, be a more common phenomenon than has previously been thought (see, Zhu *et al.*, 1994).

Although it was difficult to detect any specific binding of [^{3}H]-5-HT to CHO cell membranes other groups have detected 5-HT$_{1B}$ receptors using radioligand binding techniques both in rat brain homogenates (Hoyer *et al.*, 1985; Offord *et al.*, 1988) and in membranes from cell lines expressing native and transfected receptors (Murphy & Bylund, 1989; Voigt *et al.*, 1991; Adham *et al.*, 1992; Maroteaux *et al.*, 1992). Close examination of the data revealed a small difference between total

and non-specific binding at concentrations close to the K_D. Since the cyclase studies provided clear evidence for the presence of 5-HT$_{1B}$ receptor we concluded that this difference might indeed represent specific binding to the 5-HT$_{1B}$ receptor.

It is unlikely that our assay conditions prevented binding since they are similar to those used by Voigt *et al.* (1991) and have been used in this laboratory to detect binding to CHO cells transfected with the human 5-HT$_{1A}$ receptor gene. A more likely explanation is that the receptor density was low in our cells. In two other reports in which radioligand binding and cyclic AMP were measured in the same study (Murphy & Bylund, 1989; Adham *et al.*, 1993) agonist potency was at least 10–20 fold higher than in the present paper, consistent with a lower receptor density in our CHO cells. Indeed when Adham *et al.* (1993) used irreversible receptor alkylation with EEDQ to reduce the receptor density in Y-1 cells by up to 13 fold (7.4 and 0.55 pmol mg^{-1} protein) the specific binding was reduced to only 25% of total binding, and was difficult to quantify. If the receptor density in CHO cells is 20 fold less than in the transfected Y-1 cells then our failure to detect specific binding

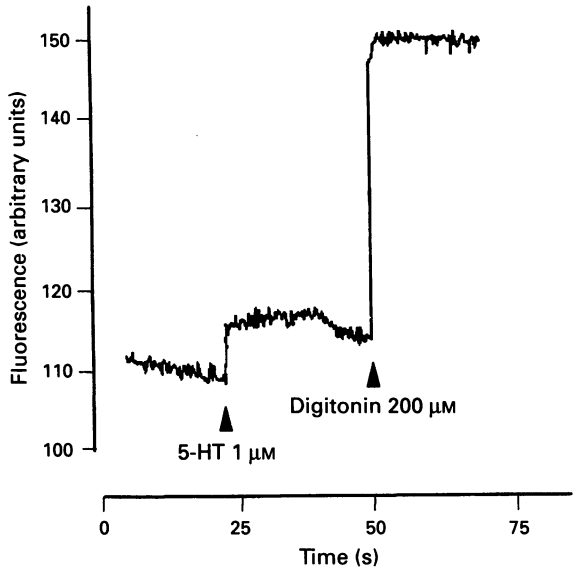

Figure 4 Representative recording showing the effect of 1 μM 5-HT on free intracellular calcium levels in CHO(dhfr-) cells loaded with the calcium-sensitive fluorescent indicator, Fura-2.

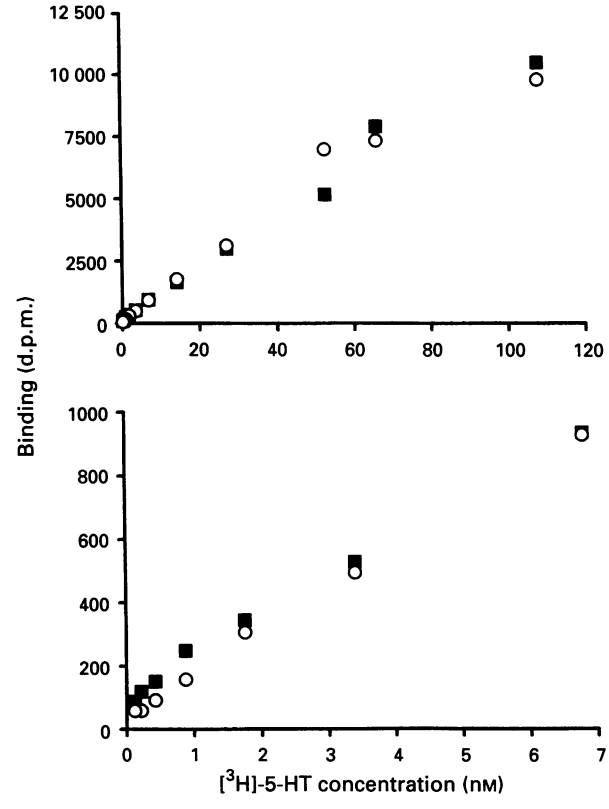

Figure 6 (a) Binding of [^{3}H]-5-HT (99 Ci mmol^{-1}) to CHO (dhfr-) cell membranes: showing total binding (■) and non-specific binding in presence of 100 μM 5-HT (○). (b) Amplification of the data in (a), showing only the lower [^{3}H]-5-HT concentrations.

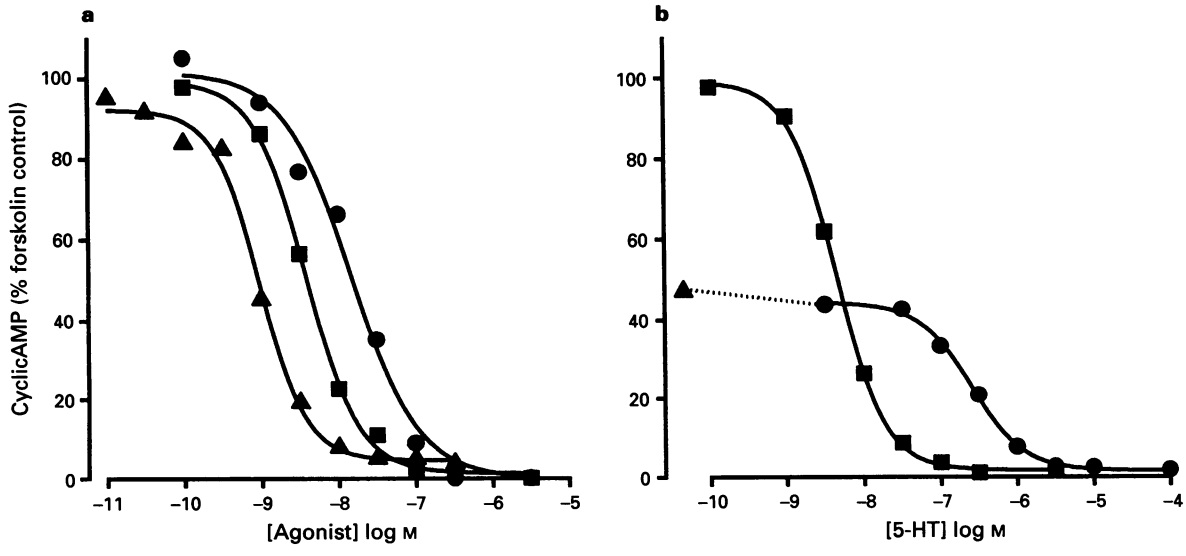

Figure 5 Agonist and antagonist studies in CHO-K1 cells. Cells were stimulated with 1 μM forskolin and responses expressed as % inhibition of the forskolin-induced increase in cyclic AMP levels. Representative data from a single day are shown. Data points are the mean of duplicates and the line through each data set is the result of fitting the individual data points to Eq.1. (a) Concentration-effect curves for 5-HT (■), RU24969 (▲) and CP-93,129 (●). (b) 5-HT concentration-effect curves in the absence (■) and presence (●) of 0.1 μM (±)-cyanopindolol. The dotted line connects the 5-HT curve in the presence of (±)-cyanopindolol to the effect of (±)-cyanopindolol alone.

can be understood. The possibility that the majority of the 5-HT$_{1B}$ receptors were in a low affinity conformation for agonist binding offers a further explanation for the absence of specific binding of 5-HT. In this regard it is interesting that Hamblin *et al.* (1992) demonstrated two affinity states for [^{3}H]-5-HT at transfected 5-HT$_{1B}$ receptors, pK_D 9.20 and 7.56, and 81% of the receptors were in the low affinity state.

Perhaps the simplest explanation for the data presented here is that CHO cells possess a low density of 5-HT$_{1B}$ receptors which are extremely well-coupled to the transduction pathway. The first step in this pathway is the binding of the receptor to the G-protein, which is accompanied by the binding of GTP. In some receptor systems considerable amplification at this step has been demonstrated, presumably because a single occupied receptor can activate several G-protein molecules (Traynor & Nahorski, 1995). We measured the binding of [^{35}S]-GTPγS to CHO cell membranes in the presence and absence of increasing concentrations of 5-HT. No increase in binding was detected, suggesting that the major step for amplification of the 5-HT$_{1B}$ response occurs after the receptor-G-protein binding step. Parallel experiments using CHO cells transfected with the 5-HT$_{1A}$ receptor resulted in agonist-dependent increases in binding, and demonstrated that the absence of binding in untransfected CHO cells was not due to incorrect assay conditions.

The presence of a native 5-HT receptor in CHO(dhfr-) cells was unexpected since many groups have previously used CHO cells as hosts for the expression of cloned 5-HT$_1$ receptors. CHO(dhfr-) cells are a mutant CHO cell line which are without the dihydrofolate reductase gene (Urlab & Chasin, 1980). We excluded the possibility that expression of the 5-HT$_{1B}$ receptor occurred at the time of the dhfr- mutation by demonstrating the presence of 5-HT$_{1B}$ receptors on CHO-K1 cells, a cell line which does not have this mutation. Numerous groups have reported the absence of [^{3}H]-5-HT binding to these cells and have therefore concluded that the cell line does not possess native 5-HT receptors. Other groups have explicitly stated that they are unable to detect any effect of 5-HT on forskolin-stimulated cyclic AMP production e.g. Cerutis *et al.* (1994). Indeed, this group then proceeded to transfect a cloned 5-HT$_{1B}$ receptor gene into CHO-K1 cells. However, others have demonstrated that 5-HT will inhibit adenylyl cyclase in their CHO cells. Van Sande *et al.* (1993) reported that 5-HT elicited inhibition of forskolin-stimulated cyclic AMP production in CHO-K1 cells but continued to transfect a human 5-HT$_{1D\alpha}$ receptor gene into this cell line. During preparation of this manuscript Berg *et al.* (1994) provided strong evidence that their CHO cells possessed 5-HT$_{1B}$ receptors capable of a functional cyclase response, although they did not report on the presence or absence of radioligand binding. The most likely explanation for the differences between groups is that there are several strains of CHO cells and that only some of these express the 5-HT$_{1B}$ receptor. However, CHO cells clearly have the potential to express functional 5-HT$_{1B}$ receptors and therefore seem an unwise choice as hosts for the transfection and expression of cloned 5-HT receptors. It is well documented that 5-HT$_1$ receptors may be present in a cell or tissue but be unable to elicit any significant response until the system is primed by the activation of another receptor e.g. contraction via 5-HT$_{1B}$ receptors in rat caudal artery can only be detected if a small amount of tone is induced in the vessel by addition of another agonist such as U46619, a thromboxane-mimetic (Craig & Martin, 1993). It is therefore quite possible that the expression of a 5-HT receptor clone in CHO cells could reveal the presence of a previously undetected native 5-HT$_{1B}$ receptor.

In conclusion, cultured CHO cells express 5-HT$_{1B}$ receptors which are negatively coupled to adenylyl cyclase and positively coupled to increases in intracellular calcium. The absence of radioligand binding was unexpected in view of the potency of 5-HT and the partial agonist activity of the normally 'silent' competitive antagonist, (±)-cyanopindolol. We can only surmise that this high efficacy is due to very efficient receptor-effector coupling of a low density of 5-HT$_{1B}$ receptors. Clearly, the absence of detectable radioligand binding cannot be assumed to mean the absence of receptors capable of eliciting a significant functional response.

We would like to thank Dr Mike Lockyer for stimulating discussion of this work, Val Hall for her help with cell culture, and Dr Dieter Riddall and Pam Fox for their assistance and advice on the radioligand binding experiments.

References

ADHAM, N., ELLERBROOK, B., HARTIG, P., WEINSHANK, R.L. & BRANCHEK, T. (1993). Receptor reserve masks partial agonist activity of drugs in a cloned 5-hydroxytryptamine$_{1B}$ system. *Mol. Pharmacol.*, **43**, 427–433.

ADHAM, N., ROMANIENKO, P., HARTIG, P., WEINSHANK, R.L. & BRANCHEK, T. (1992). The rat 5-hydroxytryptamine receptor is the species homologue of the human 5-hydroxytryptamine receptor. *Mol. Pharmacol.*, **41**, 1–7.

BERG, K.A., CLARKE, W.P., SAILSTAD, C., SALTZMAN, A. & MAAYANI, S. (1994). Signal transduction differences between 5-hydroxytryptamine type 2A and type 2C receptor systems. *Mol. Pharmacol.*, **46**, 477–484.

BLACK, J.W. & LEFF, P. (1983). Operational models of pharmacological agonism. *Proc. R. Soc.*, **220**, 141–162.

BLACK, J.W., LEFF, P. & SHANKLEY, N.P. (1985). Further analysis of anomalous pK_B values for histamine H_2 receptor antagonists on the mouse isolated stomach assay. *Br. J. Pharmacol.*, **86**, 581–587.

BOUHELAL, R., SMOUNYA, L. & BOCKAERT, J. (1988). 5-HT$_{1B}$ receptors are negatively coupled with adenylate cyclase in rat substantia nigra. *Eur. J. Pharmacol.*, **151**, 189–196.

CERUTIS, R.D., HASS, N.A., IVERSON, L.J. & BYLUND, D.B. (1994). The cloning and expression of an OK cell cDNA encoding a 5-hydroxytryptamine$_{1B}$ receptor. *Mol. Pharmacol.*, **45**, 20–28.

CRAIG, D.A. & MARTIN, G.R. (1993). 5-HT$_{1B}$ receptors mediate potent contractile responses to 5-HT in rat caudal artery. *Br. J. Pharmacol.*, **109**, 609–611.

ENGEL, G., GOTHERT, M., HOYER, D., SCHLICKER, E. & HILLENBRAND, K. (1986). Identity of pre-synaptic 5-hydroxytryptamine (5-HT) autoreceptors in the rat brain cortex with 5-HT$_{1B}$ binding sites. *Naunyn Schmied Arch. Pharmacol.*, **332**, 1–7.

HAMBLIN, M.W., McGUFFIN, R.W., METCALF, M.A., DORSA, D.M. & MERCHANT, K.M. (1992). Distinct 5-HT$_{1B}$ and 5-HT$_{1D}$ serotonin receptors in rat: structural and pharmacological comparison of the two receptors. *Mol. Cell. Neurosci.*, **3**, 578–587.

HOYER, D., ENGEL, G. & KALKMAN, H.O. (1985). Characterisation of the 5-HT$_{1B}$ recognition site in rat brain: binding studies with (-)[^{125}I]iodocyanopindolol. *Eur. J. Pharmacol.*, **118**, 1–12.

LEFF, P., PRENTICE, D.J., GILES, H., MARTIN, G.R. & WOOD, J. (1990). Estimation of agonist affinity and efficacy by direct, operational model-fitting. *J. Pharmacol. Methods*, **23**, 225–237.

MACOR, J.W., BURKHART, C.A., HEYM, J.H., IVES, J.L., LEBEL, L.A., NEWMAN, M.E., NIELSON, J.A., RYAN, K., SCHULTZ, D.W., TORGERSON, L.K. & KOE, K. (1990). 3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-b]pyrid-5-one: a potent and selective serotonin (5-HT1B) agonist and rotationally restricted phenolic analogue of 5-methoxy-3-(1,2,5,6-tetrahydropyrid-4-yl)indole. *J. Med. Chem.*, **33**, 2087–2093.

MAROTEAUX, L., SAUDOU, F., AMLAIKY, N., BOSCHERT, U., PLASSAT, J.L. & HEN, R. (1992). Mouse 5-HT$_{1B}$ serotonin receptor: cloning, functional expression, and localization in motor control centers. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3020–3024.

MAURA, G., ULIVI, M. & RAITERI, M. (1987). (-)-Propranalol and (±)-cyanopindolol are mixed agonists-antagonists at serotonin autoreceptors in the hippocampus of the rat brain. *Neuropharmacol.*, **26,** 713–717.

MURPHY, T.J. & BYLUND, D.B. (1989). Characterisation of serotonin-1B receptors negatively coupled to adenylyl cyclase in OK cells, a renal epithelial cell line from the oppossum. *J. Pharmacol. Exp. Ther.*, **249,** 535–543.

NEWMAN-TANCREDI, A., WOOTON, R. & STRANGE, P.J. (1992). High-level stable expression of recombinant 5-HT$_{1A}$ receptors in chinese hamster ovary cells. *Biochem. J.*, **285,** 933–938.

OFFORD, S.J., ORDWAT, G.A. & FRAZER, A. (1988). Application of [^{125}I]iodocyanopindolol to measure 5-hydroxytryptamine$_{1B}$ receptors in the brain of the rat. *J. Pharmacol. Exp. Ther.*, **244,** 144–153.

RAYMOND, J.R. (1991). Protein kinase C induces phosphorylation and desensitisation of the human 5-HT$_{1A}$ receptor. *J. Biol. Chem.*, **266,** 14747–14753.

SCHOEFFTER, P. & HOYER, D. (1989). 5-hydroxytryptamine 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors mediating inhibition of adenylyl cyclase activity. *Naunyn Schmied Arch. Pharmacol.*, **340,** 285–292.

SCHOEFFTER, P., PFEILSCHIFTER, J. & BOBIRNIC, I. (1994). 5-hydroxytryptamine 5-HT$_{1B}$ receptors mediate inhibition of adenylyl cyclase in a rat mesangial cell line. *Br. J. Pharmacol.*, **112,** 110P.

SUMNER, M.J., FENIUK, W., McCORMICK, J.D. & HUMPHREY, P.A. (1992). Studies on the mechanism of 5-HT$_1$ receptor-induced smooth muscle contraction in dog saphenous vein. *Br. J. Pharmacol.*, **105,** 603–608.

THOMAS, D.R., FARUQ, S.A., BALKAREK, J.M. & BROWN, A.M. (1995). Pharmacological characterisation of [^{35}S]-GTPγS binding to chinese hamster ovary cell membranes stably expressing cloned human 5-HT$_{1D}$ receptor sub-types. *J. Receptor Signal Transduction Res.*, **15,** 199–211.

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

URLAB, G. & CHASIN, L.A. (1980). Isolation of chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc. Natl. Acad. Sci. U.S.A.*, **77,** 4216–4220.

VAN SANDE, J., ALLGEIER, A., MASSART, C., CZERNILOFSKY, A., VASSART, G., DUMMONT, J.E. & MAENHAUT, C. (1993). The human and dog 5-HT$_{1D}$ receptors can both activate and inhibit adenylyl cyclase in transfected cells. *Eur. J. Pharmacol.*, **247,** 177–184.

VELDMAN, S.A. & BIENKOWSKI, M.J. (1992). Cloning and pharmacological characterisation of a novel human 5-hydroxytryptamine$_{1D}$ receptor subtype. *Mol. Pharmacol.*, **42,** 439–444.

VOIGT, M.M., LAURIE, D.J., SEEBURG, P.H. & BACH, A. (1991). Molecular cloning and characterisation of a rat brain cDNA encoding a 5-hydroxytryptamine$_{1B}$ receptor. *EMBO J.*, **10,** 4017–4023.

ZHU, X., GILBERT, S., BIRNBAUMER, M. & BIRNBAUMER, L. (1994). Dual signalling potential is common amongst G_s-coupled receptors and dependent on receptor density. *Mol. Pharmacol.*, **46,** 460–469.

(Received June 23, 1995
Revised November 22, 1995
Accepted November 23, 1995)