British Journal of Pharmacology (1998) 124, 283–290
http://www.stockton-press.co.uk/bjp

G-protein activation at 5-HT_{1A} receptors by the 5-ht_{1F} ligand LY334370 in guinea-pig brain sections and recombinant cell lines

Delphine S. Dupuis, Francis C. Colpaert & [1]Petrus J. Pauwels

Department of Cellular and Molecular Biology, Centre de Recherche Pierre Fabre, 17, avenue Jean Moulin, 81106 Castres Cédex, France

1 G-protein activation by the 5-ht_{1F} receptor agonist 5-(4-fluorobenzoyl)amino-3-(1-methylpiperidin-4-yl)-1H-indole fumarate (LY334370) was investigated by use of autoradiography of receptor-activated G-proteins in guinea-pig brain sections and [^{35}S]-GTPγS binding responses in cell lines stably expressing human 5-HT_{1A} (h 5-HT_{1A}) receptors.

2 LY334370 (10 μM) caused little or no stimulation of [^{35}S]-GTPγS binding in guinea-pig brain regions enriched in 5-ht_{1F} binding sites (e.g., claustrum, caudate/putamen and thalamic nuclei), as identified by labelling with 10 nM [^{3}H]-sumatriptan plus 10 nM 5-carboxamidotryptamine (5-CT).

3 Application of LY334370 (10 μM) to guinea-pig brain sections resulted in an increase of [^{35}S]-GTPγS binding in hippocampus (123$\pm$17%), lateral septum (58$\pm$14%), dorsal raphe (57$\pm$10%), entorhinal (37$\pm$11%) and cingulate cortex (28$\pm$10%). This distribution fits with the G-protein activation mediated by 5-HT_{1A} receptors as found with lisuride (10 μM), and labelling of 5-HT_{1A} receptors by 140 pM [^{125}I]-4-(2′-methoxy-phenyl)-1-[2′-(n-2″-pyridinyl)-p-iodobenzamido]-ethyl-piperazine (p-MPPI).

4 The LY334370-mediated [^{35}S]-GTPγS response was antagonized by the selective, silent 5-HT_{1A} receptor antagonist N-[2-[4-(2-methoxyphenyl)1-piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexanecarboxamide (WAY100635, 1 μM) in each of the brain structures investigated. The distribution pattern of the [^{35}S]-GTPγS binding response and the antagonist profile suggest that the LY334370-induced response in guinea-pig brain is mediated by 5-HT_{1A} receptors.

5 The maximal LY334370-induced [^{35}S]-GTPγS binding response (83 to 94%) in membranes of recombinant C6-glial/h 5-HT_{1A} and HeLa/h 5-HT_{1A} cells was close to that of 5-HT, suggesting LY334370 to exert high intrinsic activity at h 5-HT_{1A} receptors.

6 In conclusion, in guinea-pig brain sections and recombinant cell lines the 5-ht_{1F} receptor agonist LY334370 causes G-protein activation that is mediated by 5-HT_{1A} receptors. Caution should be taken when employing this ligand as a putative selective 5-ht_{1F} agonist.

Keywords: 5-ht_{1F} and 5-HT_{1A} receptor; G-protein activation; [^{35}S]-GTPγS binding and autoradiography; guinea-pig brain; recombinant h 5-HT_{1A} cell lines

Introduction

5-Hydroxytryptamine (5-HT) elicits diverse physiological responses as a neurotransmitter or neuromodulator in the mammalian central nervous system through multiple distinct receptor subtypes (see Hoyer *et al*., 1994). Of these, 5-ht_{1F} receptors have received particular attention because of their putative involvement in the neurogenic dural inflammation model of migraine (Johnson *et al*., 1996; Phebus *et al*., 1996). This presumed involvement is based mainly on observations made with 5-(4-fluorobenzoyl)amino-3- (1-methylpiperidin-4-yl)- 1H-indole fumarate (LY334370), an apparently selective high affinity agonist at the 5-ht_{1F} receptor (Lucaites *et al*., 1996; Overshiner *et al*., 1996; Wainscott *et al*., 1996). LY334370 also shows nanomolar binding affinity for the 5-HT_{1A} receptor, but *in vivo* studies have so far failed to reveal any functional 5-HT_{1A} agonist or antagonist activity of LY334370 at doses that are several orders of magnitude higher than that (i.e., 0.1 mg kg^{-1}, s.c.) at which the compound is fully effective in the 5-ht_{1F}-sensitive rat dural extravasation model (Overshiner *et al*., 1996).

The aim of the present study was to explore G-protein activation mediated by 5-ht_{1F} binding sites in native guinea-pig brain tissue. Autoradiography of 5-ht_{1F} receptor-activated G-proteins was determined by agonist-stimulated [^{35}S]-GTPγS binding. This approach provides a method of functional neuroanatomy that identified changes in the activation of G-proteins by μ-opioid, cannabinnoid, γ-aminobutyric acid type B and 5-$HT_{1A/B/D}$ receptors (Sim *et al*., 1995; Waeber & Moskowitz, 1996; Scott & Bruinvels, 1997; Stanton *et al*., 1997; Dupuis *et al*., 1998). The experiments analysed the relationship between the 5-ht_{1F} binding site distribution obtained by receptor binding autoradiography and the activated state of G-proteins by LY334370. The data failed to reveal any substantial activation by LY334370 of G-proteins in guinea-pig brain regions rich in 5-ht_{1F} binding sites, while the compound did cause G-protein activation at 5-HT_{1A} receptors. The activity of LY334370 was further explored in membrane preparations of cell lines stably expressing human 5-HT_{1A} (h 5-HT_{1A}) receptors.

Methods

Preparation of guinea-pig brains sections

Male Hartley guinea-pigs (300–350 g) were killed by decapitation; the whole brain was removed and frozen in isopentane cooled at -35°C. The brain was sectioned into 20 μm thick coronal (anterior 10.6 to 4.2 mm; Luparello, 1967) and horizontal brain sections (4th ventricle to the aqueduct) with a cryostat-microtome (Leica JUNG CM 3000) at -20°C. Sections were thawed-mounted on adhesive

[1] Author for correspondence.

microscope slides, dried under vacuum overnight and used fresh or stored at −80°C for maximally one month before use.

5-ht$_{1F}$ and 5-HT$_{1A}$ receptor binding autoradiography to guinea-pig brain sections

Incubations were performed principally as previously described (Waeber & Moskowitz, 1995; Kung *et al*., 1995). Briefly, sections were preincubated for 20 min at 25°C in Krebs solution (composition in mM: NaCl 118, KCl 4.8, $CaCl_2$ 1.2, $MgCl_2$ 1.2 and Tris-HCl 15; pH 7.4), and then covered with 0.5 ml of Krebs solution containing 10 nM [^{3}H]-sumatriptan and 10 nM 5-CT for 1 h to label 5-ht$_{1F}$ binding sites. Non-specific binding was determined in the presence of 10 μM 5-HT. The incubation was stopped by two washes in ice-cold 50 mM Tris-HCl buffer (pH 7.4) and a dip in ice-cold deionized water. Sections were dried under a stream of cold air and then exposed to Kodak Biomax MR film for a period of between 4 to 6 weeks. For [^{125}I]-p-MPPI autoradiography of 5-HT$_{1A}$ receptors, sections were exposed to 140 pM of [^{125}I]-4-(2′-methoxy-phenyl)-1-[2′-(n-2″-pyridinyl)-p-iodobenzamido]-ethyl-piperazine ([^{125}I]-p-MPPI) for 2 h. Non-specific binding was determined in the presence of 10 μM 8-(hydroxy)-2-(di-n-propylanime) tetralin (8-OH-DPAT). The incubation was stopped by two washes for 30 min in ice-cold 50 mM Tris-HCl buffer (pH 7.4), followed by a dip in ice-cold deionized water. Sections were exposed to film between 12 to 60 h.

Autoradiography of 5-ht$_{1F}$ and 5-HT$_{1A}$ receptor agonist-stimulated [^{35}]-GTPγS binding to guinea-pig brain sections

Autoradiography of 5-ht$_{1F}$ receptor-stimulated [^{35}S]-guanosine-5′-*O*-(γ-thiotriphosphate) ([^{35}S]-GTPγS binding in brain sections was performed principally as described by Sim *et al*. (1995). Sections were preincubated for 10 min at 25°C in 50 mM Tris-HCl supplemented with 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl (pH 7.4). Thereafter, sections were exposed to 2 mM guanosine 5′-diphosphate (GDP) for 20 min and subsequently exposed to Tris-HCl buffer containing 2 mM GDP and 50 pM [^{35}S]-GTPγS either in the absence or presence of LY334370 for 2 h at 25°C. Basal [^{35}S]-GTPγS binding was defined in the absence of agonist. The incubation was stopped by two washes with ice-cold 50 mM Tris-HCl (pH 7.4), and a brief immersion in ice-cold deionized water. Sections were dried and exposed to Kodak Biomax MR film as described above. Autoradiography of 5-HT$_{1A}$ receptor agonist-stimulated [^{35}S]-GTPγS binding was performed in the presence or absence of antagonist as described by Dupuis *et al*. (1998), with lisuride instead of L694247.

Cell lines with stable expression of human 5-HT$_{1A}$ receptors

The HeLa/HA7 cell line containing the pRK7/h 5-HT$_{1A}$ plasmid and stably expressing h 5-HT$_{1A}$ receptors (Fargin *et al*., 1989) was cultured as previously described (Pauwels *et al*., 1993). C6-glial cells stably transfected with a pcDNA3/h 5-HT$_{1A}$ plasmid (Wurch *et al*., 1996) were cultured as described for C6-glial/h 5-HT$_{1B}$ cells (Pauwels *et al*., 1996). h 5-HT$_{1A}$ receptor binding was performed as previously described with [^{3}H] 8-OH-DPAT (Pauwels *et al*., 1993).

[^{35}S]-GTPγS binding responses with membrane preparations of h 5-HT$_{1A}$ receptor transfected C6-glial and HeLa cell lines

Agonist-stimulated [^{35}S]-GTPγS binding was examined as previously described (Pauwels *et al*., 1997). Briefly, C6-glial and HeLa membranes (16 to 28 μg and 38 to 90 μg of protein, respectively) were preincubated for 30 min at 25°C in 20 mM HEPES (pH 7.4) supplemented with the indicated concentrations of GDP, 100 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM ascorbic acid, in either the absence or presence of LY334370 or 8-OH-DPAT. [^{35}S]-GTPγS (500 pM) was subsequently added for 30 min. Maximal stimulation of [^{35}S]-GTPγS binding was defined in the presence of 10 μM 5-HT. E_{max} values were expressed as a percentage of the maximal response obtained with 10 μM 5-HT. EC_{50} values were defined as the concentration of compound at which 50% of its own maximal stimulation was obtained. In antagonist experiments, WAY100635 was co-incubated with LY334370. pK_B values were calculated as $K_B = (B)/(A'/A) - 1$ where B is the concentration of the antagonist, and A and A′ are the EC_{50} values of agonist concentration measured in the absence and presence of antagonist. Membrane protein levels were estimated with the dye-binding assay by means of the Bio-Rad kit (Bradford, 1976). Bovine serum albumin was used as a standard.

Data analysis and statistics

Images were densitometrically analysed with a microcomputer-based image analysis system (Imagena 2000 Biocom, Les Ulis, France). Optical densities were transformed into levels of bound radioactivity (fmol mg^{-1} tissue equivalent) with grey-values generated by coexposed ^{3}H, ^{125}I and ^{14}C polymer standards. For tritiated and iodinated radioligands, specific binding values were obtained by substracting non-specific binding values from total binding values. For [^{35}S]-GTPγS binding, [^{35}S]-GTPγS binding data were analysed with a ^{14}C Microscale, and corrected for the specific activity of [^{35}S]-GTPγS at the calibration date and the decay factor of ^{35}S. Antagonist data were expressed as a percentage of the maximal response obtained with 10 μM LY334370. Quantified data were obtained from 4 to 6 independent brains, and sections were each time run in duplicate. Statistical analysis was performed on the [^{35}S]-GTPγS E_{max} values expressed versus that obtained with LY334370 from the right side of the sections, by means of the non-parametric Friedman's two way ANOVA-test. Similar results were obtained with the left side of the sections. For the [^{35}S]-GTPγS binding responses on cell lines, statistical analysis was performed on the E_{max} values of LY334370 and 8-OH-DPAT expressed *versus* 10 μM 5-HT, by use of the non-parametric Mann-Whitney U test.

Materials

SuperFrost/Plus adhesive microscope slides were obtained from O. Kindler Gmbh & Co (Freiburg, Germany). Autoradiographic ^{3}H (0.1–16 nCi mg^{-1}; 3–110 nCi mg^{-1}), ^{125}I (1.25–640 nCi mg^{-1}) and ^{14}C (31–883 nCi g^{-1}) microscales, and Kodak Biomax MR Film were obtained from Amersham (Les Ulis, France). [^{125}I]-p-MPPI, (2200 Ci mmol^{-1}), [^{35}S]-GTPγS (1000 to 1103 Ci mmol^{-1}), [^{3}H]-sumatriptan (81 Ci mmol^{-1}) and [^{3}H]-8-OH-DPAT (217 to 228 Ci mmol^{-1}) were obtained from Amersham (Les Ulis, France). The HeLa/HA7 cell line was obtained from Tulco (Duke University, Durham, NC, U.S.A.). C6-glial cells were

obtained from ATCC (Rockville, U.S.A.). Cell culture, media, foetal calf serum, culture plates were obtained from Gibco Biocult Laboratories (Paisley, U.K.). The Emulsifier-Safe was obtained from Packard (Warrenville, PA, U.S.A.). 5-HT was from Sigma (St Louis, U.S.A.). Racemic 8-(hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), 5-CT and ketanserin were from RBI (Natick, U.S.A.). L694247 and methiothepin were obtained from Tocris Cookson (Bristol, U.K.). Lisuride was a gift from Schering (Berlin, Germany). (2′-Methyl-4′-(5-methyl[1,2,4]oxadiazol-3-yl)biphenyl-4-carboxylic acid [4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]amide (GR127935), 1′-methyl-5-(2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carbonyl)-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4′-piperidine] (SB224289), LY334370 and WAY100635 were synthesized at the Centre de Recherche Pierre Fabre.

Results

Comparison between the [^{35}S]-GTPγS binding response induced by LY334370 and the distribution of 5-ht$_{1F}$ and 5-HT$_{1A}$ receptors in guinea-pig brain sections

In order to measure G-protein activation by the 5-ht$_{1F}$ agonist LY334370 in guinea-pig brain, an initial series of autoradiographic experiments was performed with [^{3}H]-sumatriptan to localize 5-ht$_{1F}$ binding sites in coronal brain sections displayed in a rostro-caudal progression. [^{3}H]-sumatriptan labels two populations of 5-HT$_1$ binding sites; one of these displays nanomolar affinity for 5-CT which appears similar to that of the 5-HT$_{1B/D}$ radioligand [^{125}I]-GTI (Waeber & Moskowitz, 1995). These binding sites were absent in the presence of 10 nM 5-CT while 5-ht$_{1F}$ binding sites, which have low affinity for 5-CT (Adham *et al*., 1993), were still visualized. This labelling was mainly present in the caudate/putamen (Figure 1, A1), claustrum (Figure 1, A1 and A2), intermediate cortical layer (Figure 1, A1 to A3), dorsolateral geniculate nucleus, (Figure 1, A2) and medial mammillary nucleus (Figure 1, A3). Figure 1 (B1 to B9) also shows the [^{35}S]-GTPγS binding response to this series of coronal brain sections. In comparison with the basal condition (Figure 2, B1 to B3), application of LY334370 (10 μM) resulted in an increase of [^{35}S]-GTPγS binding in the lateral septum, hippocampus, dorsal raphe, superficial grey layer of superior colliculi, interpeduncular nucleus and entorhinal cortex (Figure 2, B4 to B6). This distribution fits with the G-protein activation being mediated by 5-HT$_{1A}$ receptors, as shown with lisuride (10 μM, Figure 2, B7 to B9) and with the labelling of 5-HT$_{1A}$ receptors by 140 pM [^{125}I]-p-MPPI (Figure 2, C1 to C3). However, LY334370 caused little or no stimulation of [^{35}S]-GTPγS binding in brain regions enriched in 5-ht$_{1F}$ binding sites (e.g., claustrum, caudate/putamen and thalamic nuclei). Similar findings were obtained with horizontal guinea-pig brain sections (Figure 2). On these sections, 5-ht$_{1F}$ binding sites were localized in the claustrum, caudate/putamen and intermediate cortical layer (Figure 2, A1). [^{35}S]-GTPγS binding obtained in the presence of either LY334370 (10 μM, Figure 2, B1) or lisuride (10 μM, Figure 2, B2) was identified in brain regions (e.g. lateral septum, hippocampus, dorsal raphe, cingulate and entorhinal cortex) labelled with [^{125}I]-p-MPPI (Figure 2, C1). Quantitative assessment of the [^{35}S]-GTPγS binding response shows that the LY334370-induced response was larger in the hippocampus (123±17%) than in the lateral septum (58±14%), dorsal raphe (57±10%), entorhinal (37±11%) and cingulate cortex (28±10%). The maximal response to LY334370 in each of these brain regions was between 69 to 102% compared to that produced by lisuride (10 μM). Figure 3 illustrates the LY334370-mediated [^{35}S]-GTPγS binding response in the presence of various 5-HT receptor antagonists. The silent 5-HT$_{1A}$ receptor antagonist WAY100635 (1 μM) and the nonselective 5-HT antagonist methiothepin (1 μM) fully antagonized the [^{35}S]-GTPγS response to LY334370 in each of the brain regions investigated. The 5-HT$_{1B}$ inverse agonist SB224289 (1 μM), the 5-HT$_{1B/D}$ antagonist GR127935 (1 μM)

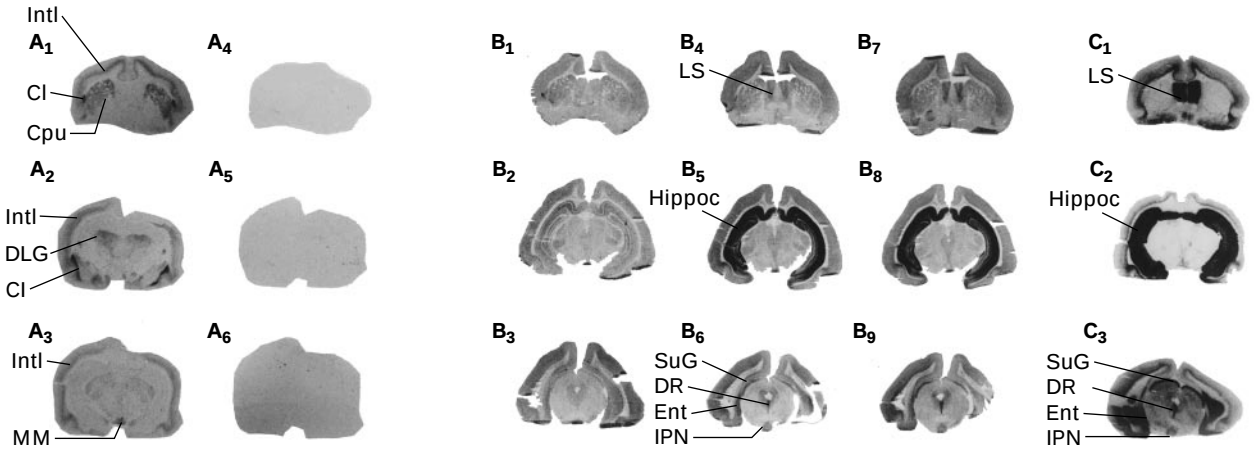

Figure 1 Autoradiograms of coronal guinea-pig brain sections at three different levels of anterior position (see Methods) exposed to 10 nM [^{3}H]-sumatriptan (plus 10 nM 5-CT) either in the absence (A_1 to A_3) or presence of 10 μM 5-HT (A_4 to A_6), 50 pM [^{35}S]-GTPγS and 2 mM GDP in either the absence (basal, B_1 to B_3) or presence of 10 μM LY334370 (B_4 to B_6) or lisuride (B_7 to B_9), and 140 pM [^{125}I]-p-MPPI (C_1 to C_3). [^{3}H]-sumatriptan (plus 10 nM 5-CT) binding sites are observed in claustrum (A_1, A_2), caudate/putamen (A_1), intermediate cortical layers (A_1, A_2, A_3), dorsolateral geniculate nucleus (A_2) and medial mammillary nucleus (A_3). LY334370 and lisuride-mediated [^{35}S]-GTPγS binding was present in lateral septum (B_4, B_7), hippocampus (B_5, B_8), superficial grey layer of superior colliculi, interpeduncular nucleus, dorsal raphe and entorhinal cortex (B_6, B_9). [^{125}I]-p-MPPI binding was localized in lateral septum (C_1), hippocampus (C_2), superficial grey layer of superior colliculi, interpeduncular nucleus, dorsal raphe and entorhinal cortex (C_3). Non-specific [^{125}I]-p-MPPI binding (in the presence of 10 μM 8-OH-DPAT) was extremely low and is not shown. Cl, claustrum; Cpu, caudate/putamen; Intl, intermediate cortical layer; DLG, dorsolateral geniculate nucleus; MM, medial mammillary nucleus; LS, lateral septum; Hippoc, hippocampus; SuG, superficial grey layer of superior colliculi; IPN, interpeduncular nucleus; DR, dorsal raphe; Ent, entorhinal cortex.

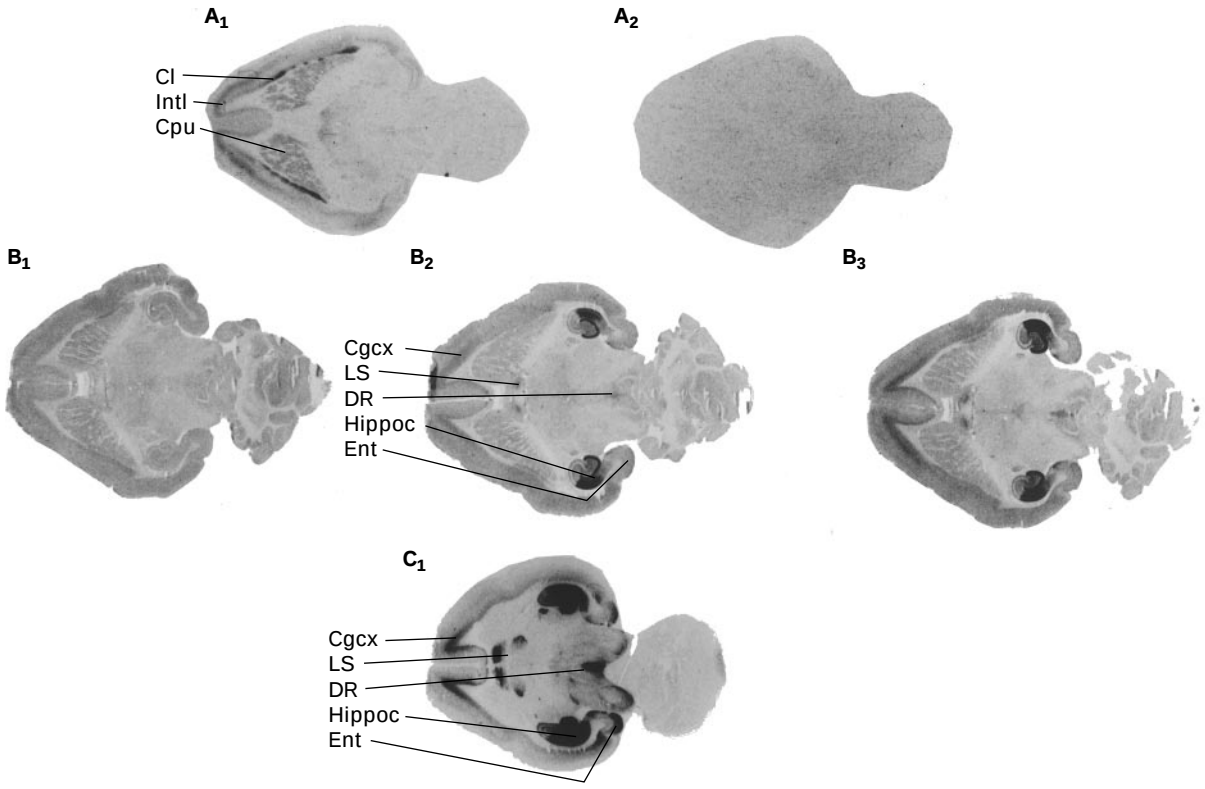

Figure 2 Comparison between labelling of horizontal guinea-pig brain sections by [^{3}H]-sumatriptan (plus 10 nM 5-CT), [^{125}I]-p-MPPI and [^{35}S]-GTPγS in the presence of LY334370 and lisuride. Sections were exposed to radioligands as described in the legend of Figure 1. [^{3}H] sumatriptan binding sites in the presence of 10 nM 5-CT were observed in claustrum, caudate/putamen and intermediate cortical layer (A_1). Non-specific [^{3}H]-sumatriptan binding in the presence of 10 μM 5-HT is shown in A_2. LY334370 (B_2) and lisuride (B_3) mediated [^{35}S]-GTPγS binding was observed in cingulate and entorhinal cortex, lateral septum, hippocampus and dorsal raphe. B_1 corresponds to basal [^{35}S]-GTPγS binding. [^{125}I]-p-MPPI binding sites were present in the same brain structures (C_1) as found for [^{35}S]-GTPγS binding with LY334370 and lisuride. Non-specific [I^{125}]-p-MPPI binding was extremely low and is not shown. Cgcx, cingulate cortex. Other abbreviations are as indicated in the legend of Figure 1.

and the 5-HT$_{2A}$/5-HT$_{1D}$ antagonist ketanserin (1 μM) did not affect the LY334370-mediated response. Each of these antagonists was without effect at 1 μM on basal [^{35}S]-GTPγS binding. Figure 4 shows the quantitative data for antagonism of the LY334370-mediated [^{35}S]-GTPγS binding response in the hippocampus. Similar results were obtained in the dorsal raphe and lateral septum (not shown).

LY334370-mediated [^{35}S]-GTPγS binding responses in membrane preparations containing recombinant human 5-HT$_{1A}$ receptors

Membrane preparations of C6-glial and HeLa cells stably transfected with a h 5-HT$_{1A}$ receptor were used to investigate the LY334370-mediated [^{35}S]-GTPγS binding response. We previously observed that the maximal [^{35}S]-GTPγS binding response of partial 5-HT$_{1A}$ receptor agonists in C6-glial cells was attenuated by increasing the GDP concentration (Pauwels *et al*., 1997). Consequently, [^{35}S]-GTPγS binding responses for LY334370 were performed at either 0.3 and/or 30 μM GDP. Figure 5 compares the concentration binding curves for stimulation of [^{35}S]-GTPγS binding for LY334370 and 8-OH-DPAT. The corresponding E_{max} and pEC_{50} values are summarized in Table 1, and compared with their pK_i values for the h 5-HT$_{1A}$ receptor. The potencies and maximal effects were similar for both ligands in HeLa and C6-glial membranes at 30 and 0.3 μM GDP, respectively. Otherwise, the maximal effect of 8-OH-DPAT in C6-glial membranes was attenuated from 90 to 41% ($P<0.01$) by increasing the GDP concentration from 0.3 to 30 μM, respectively. In contrast, the maximal effect of LY334370 was slightly increased (+11%, $P<0.05$) at 30 μM GDP. WAY100635 (10 nM) competitively antagonized the LY334370-mediated [^{35}S]-GTPγS binding response (Figure 5c); this was observed with a pK_B value of between 9.49 (C6-glial, 0.3 μM GDP) and 10.18 (HeLa, 30 μM GDP).

Discussion

The data provide evidence that the 5-ht$_{1F}$ ligand LY334370 causes activation of G-proteins via 5-HT$_{1A}$ receptors. This was at first investigated in guinea-pig brain sections by agonist-stimulated [^{35}S]-GTPγS binding. In contrast to structures enriched in 5-ht$_{1F}$ binding sites in the guinea-pig brain (e.g., claustrum, caudate/putamen and thalamic nuclei), G-protein activation by LY334370 was observed in brain regions containing 5-HT$_{1A}$ receptors (i.e., hippocampus, lateral septum, dorsal raphe, superficial grey layer of superior colliculi, interpeduncular nucleus, entorhinal and cingulate cortex). The intrinsic activity of LY334370 was further determined at the h 5-HT$_{1A}$ receptor in recombinant C6-glial and HeLa cell lines. Whereas LY334370 was slightly less potent than 8-OH-DPAT at h 5-HT$_{1A}$ receptors, its maximal effect was similar to or greater than that obtained with 8-OH-DPAT. Below we will discuss the following three points: the apparent lack of activation of G-proteins in guinea-pig brain sections upon stimulation of 5-ht$_{1F}$ binding sites, activation of G-proteins by LY334370 via 5-HT$_{1A}$ receptors, and the

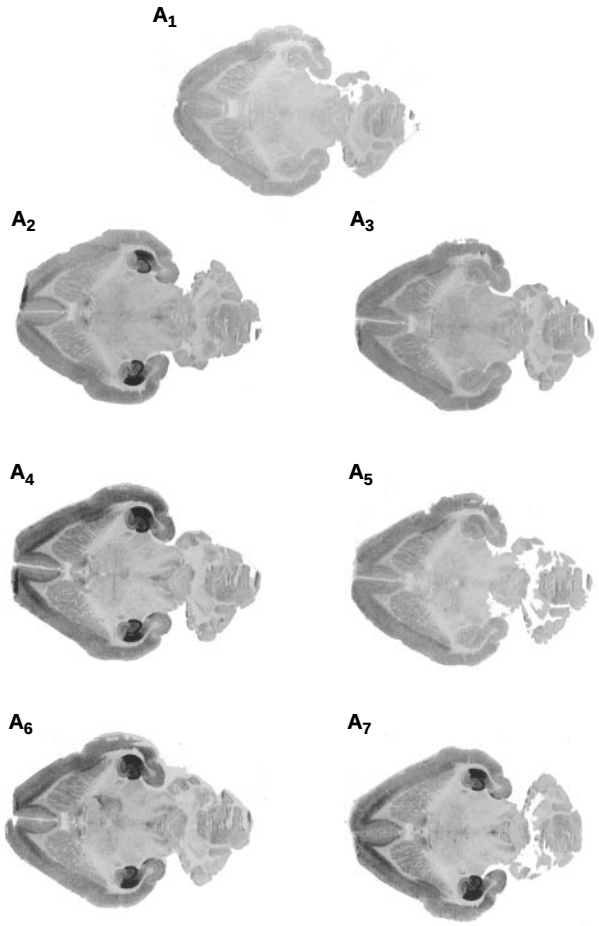

Figure 3 Blockade of LY334370-mediated [^{35}S]-GTPγS binding response to horizontal guinea-pig brain sections by WAY100635, SB224289, methiothepin, GR127935 and ketanserin. Sections were incubated with 50 pM [^{35}S]-GTPγS, 2 mM GDP in either the absence (basal condition, A_1) or presence of 10 μM LY334370 without addition (A_2), or plus 1 μM WAY100635 (A_3), SB224289 (A_4), methiothepin (A_5), GR127935 (A_6) or ketanserin (A_7).

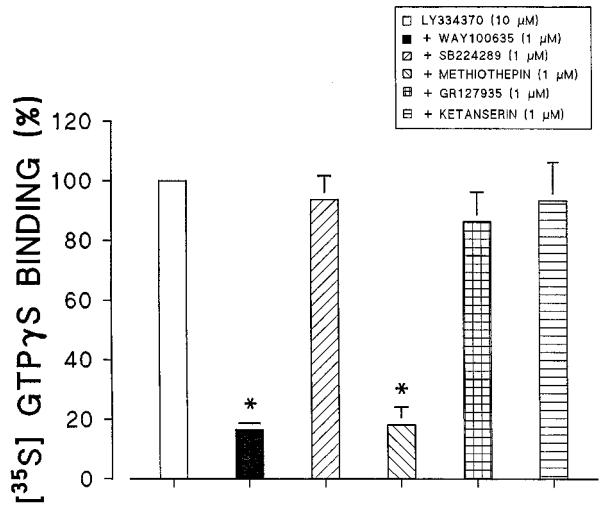

Figure 4 Quantitative assessment of blockade of LY334370-mediated [^{35}S]-GTPγS binding to hippocampus by WAY100635, SB224289, methiothepin, GR127935, and ketanserin. Horizontal guinea-pig brain sections were incubated with 50 pM [^{35}S]-GTPγS, 2 mM GDP and 10 μM LY334370 in either the absence or presence of 1 μM WAY100635, SB224289, methiothepin, GR127935, or ketanserin. Quantification of autoradiograms was performed as described in Methods. Stimulation of [^{35}S]-GTPγS binding is expressed as a percentage of the stimulation obtained with 10 μM LY334370. Columns represent mean values $\pm$ s.e.mean from 4 independent experiments, each one performed in duplicate. $^{*}P<0.05$ *vs* 10 μM LY334370 (non-parametric Friedman's two way ANOVA-test).

intrinsic activity of LY334370 at 5-HT$_{1A}$ receptors compared to that of 5-HT$_{1A}$ receptor agonists.

Apparent lack of activation of G-proteins in guinea-pig brain upon stimulation of 5-ht$_{1F}$ binding sites with LY334370

LY334370 was used in this study as an agonist of 5-ht$_{1F}$ binding sites (Lucaites *et al*., 1996; Overshiner *et al*., 1996; Wainscott *et al*., 1996). The autoradiographic guinea-pig data show that the LY334370-mediated [^{35}S]-GTPγS binding response did not correspond to the distribution of 5-ht$_{1F}$ binding sites labelled by [^{3}H]-sumatriptan in the presence of 10 nM 5-CT. 5-ht$_{1F}$ binding sites are enriched in claustrum, caudate/putamen and thalamic nuclei in accordance with the distribution pattern of these binding sites in guinea-pig, as measured with [^{3}H]-5-HT plus 100 nM 5-CT and 300 nM mesulergine (Beer *et al*., 1993; Stanton *et al*., 1996) or with [^{3}H] sumatriptan plus 10 nM 5-CT (Waeber & Moskowitz, 1995; Mengod *et al*., 1996) and [^{3}H]-LY334370 (Lucaites *et al*., 1996). Whereas the distribution of 5-ht$_{1F}$ binding sites in brain is species dependent (Beer *et al*., 1993), these sites are highly enriched in the claustrum of guinea-pig (67 fmol mg^{-1} tissue equivalent, Stanton *et al*., 1996). This is comparable with the density of 5-HT$_{1A}$ receptors in lateral septum (40 fmol mg^{-1} tissue equivalent, Sijbesma *et al*., 1991), a region sensitive to G-protein activation by 5-HT$_{1A}$ receptor agonists (Dupuis *et al*., 1998). Therefore, the observed 5-ht$_{1F}$ receptor binding density is probably not related to the apparent lack of G-protein activation by LY334370. The discrepancy between 5-ht$_{1F}$ receptor binding and receptor-mediated G-protein activation suggests a low catalytic amplification factor for 5-ht$_{1F}$ binding sites. Sim *et al*. (1996) suggested that different G-protein coupled receptors may have different intrinsic abilities to catalytically activate G-proteins. G-protein activation mediated by 5-HT$_{1A}$ receptors can apparently be monitored in rat hippocampus (167%, Scott & Bruinvels, 1997; Stanton *et al*., 1997) and in guinea-pig hippocampus (200%, Waeber & Moskowitz 1996; 140%, Dupuis *et al*., 1998). Otherwise, this is less evident for 5-HT$_{1B/D}$ receptors; 30 to 109% stimulation of [^{35}S]-GTPγS binding with 5-HT$_{1B/D}$ agonists has been observed in rat and guinea-pig substantia nigra, although antagonism of this effect was either absent or not investigated (Waeber & Moskowitz 1996; Scott & Bruinvels 1997; Stanton *et al*., 1997). Different receptors may couple to different types of G-protein subunits and their relative affinities for GDP and GTPγS may vary; as a result the efficacy of receptor G-protein activation in [^{35}S]-GTPγS binding responses may also vary (Sim *et al*., 1996).

Activation of G-proteins by LY334370 via 5-HT$_{1A}$ receptors in guinea-pig brain

Stimulation of [^{35}S]-GTPγS binding with LY334370 was measured in guinea-pig brain regions containing 5-HT$_{1A}$ receptors, such as hippocampus, lateral septum, dorsal raphe, entorhinal and cingulate cortex. This distribution pattern corresponds with the autoradiographic labelling of 5-HT$_{1A}$ binding sites by [^{125}I]-p-MPPI (Kung *et al*., 1995; Dupuis *et al*., 1998), [^{3}H]-8-OH-DPAT (Vergé *et al*., 1986), [^{125}I]-BH-8-MeO-NPAT (Gozlan *et al*., 1988) and [^{3}H]-5-methyl-urapidil (Laporte *et al*., 1991). The LY334370-mediated [^{35}S]-GTPγS

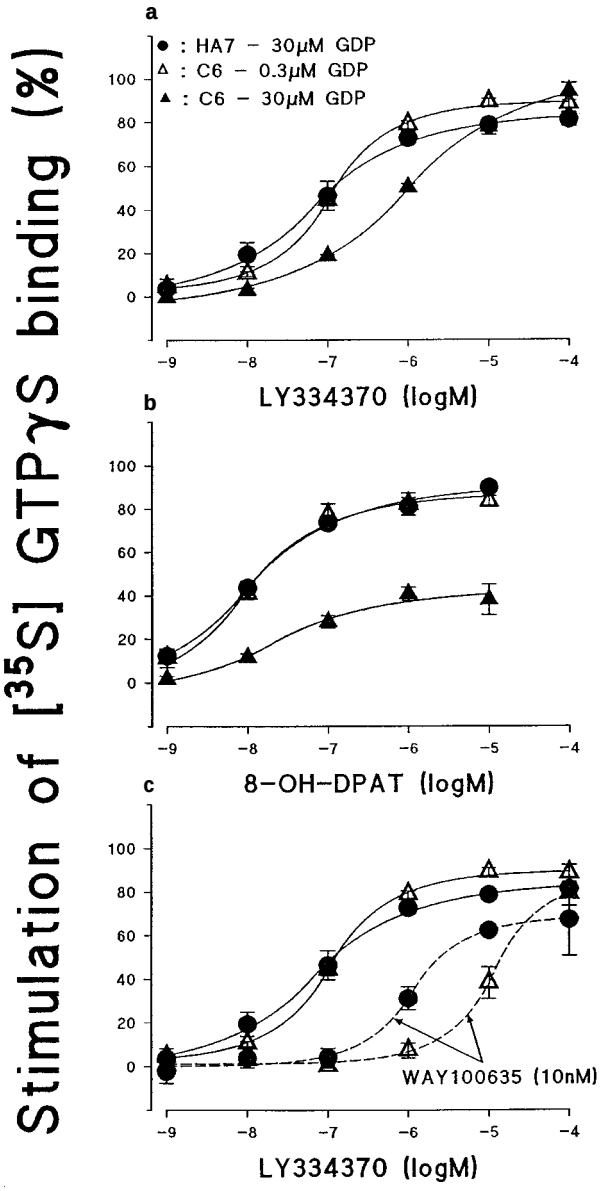

Figure 5 Concentration binding curves of LY334370 and 8-OH-DPAT for stimulation of [^{35}S]-GTPγS binding to C6-glial and HeLa membranes with h 5-HT$_{1A}$ receptors. Binding was performed in the presence of either 0.3 and/or 30 μM GDP as described in Methods. Stimulation of [^{35}S]-GTPγS binding is expressed as a percentage of the stimulation obtained with 10 μM 5-HT at the corresponding GDP concentration. Each point represents the mean of 3 to 6 independent experiments, each one performed in triplicate and vertical lines indicate s.e.mean. Mean E_{max} and pEC_{50} are summarized in Table 1. (a) LY334370, (b) 8-OH-DPAT, (c) LY334370 in either the absence or presence of WAY100635 (10 nM).

response also fits with that of activation of 5-HT$_{1A}$ receptors by lisuride (this study), L694247, 8-OH-DPAT and flesinoxan (Dupuis *et al*., 1998). Moreover, the antagonism of the LY334370-mediated [^{35}S]-GTPγS binding responses by the selective, silent 5-HT$_{1A}$ antagonist WAY100635 is further evidence that this response is mediated by 5-HT$_{1A}$ receptors. In contrast, the 5-HT$_{1B/D}$ antagonist GR127935, the selective 5-HT$_{1B}$ inverse agonist SB224289 and the 5-HT$_{2A/1D}$ antagonist ketanserin were without effect. Hence, the distribution pattern of the [^{35}S]-GTPγS binding response and the antagonist profile suggest that the LY334370-induced response in guinea-pig brain is mediated by 5-HT$_{1A}$ receptors.

Intrinsic activity of LY334370 at 5-HT$_{1A}$ receptors compared to 5-HT$_{1A}$ receptor agonists

The maximal [^{35}S]-GTPγS binding responses to LY334370 observed here were similar to those obtained with lisuride (this study) and L694247 (Dupuis *et al*., 1998). This suggests that under the present experimental conditions LY334370 is an efficacious 5-HT$_{1A}$ agonist in native guinea-pig brain tissue. This was further confirmed at h 5-HT$_{1A}$ receptors in recombinant cell lines. Overshiner *et al*. (1996) observed a low potency (pEC_{50}: 5.81) but large response to 0.98 for LY334370 (relative to 5-HT = 1.00) by measuring inhibition of forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclicAMP) production. LY334370 was more potent in our [^{35}S]-GTPγS binding response, its pEC_{50} (i.e., 7.15 to 7.24) being only 4 times lower than its pK_i value for h 5-HT$_{1A}$ receptors (this study, Overshiner *et al*., 1996). The potency of LY334370 was also close to that of 8-OH-DPAT (pEC_{50}: 7.98). The respective maximal [^{35}S]-GTPγS responses to LY334370 (E_{max}: 83 to 94%) were similar to those to L694247 (E_{max}: 84 to 98%, Pauwels *et al*., 1997), and represent high efficacy at h 5-HT$_{1A}$ receptors. Whereas the maximal response to either of these latter ligands was not attenuated by increasing the GDP concentration, this was clearly the case for the partial agonists 8-OH-DPAT and its enantiomers, flesinoxan, buspirone, spiroxatrine and ipsapirone (Pauwels *et al*., 1997). Therefore, LY334370 is efficacious at h 5HT$_{1A}$ receptors and its maximal effect is apparently not attenuated by GDP.

In conclusion, G-protein activation by 5-HT$_{1A}$ and not 5-ht$_{1F}$ receptors has been measured with LY334370 in guinea-pig brain sections and recombinant cell lines. LY334370 in fact appears to possess higher efficacy than 8-OH-DPAT at the 5-HT$_{1A}$ receptor. Nevertheless, it remains unclear why LY334370 is apparently devoid of agonist or antagonist activity at 5-HT$_{1A}$ receptors in *in vivo* experimental conditions (Overshiner *et al*., 1996).

Table 1 E_{max} and pEC_{50} values of LY334370 and 8-OH-DPAT for stimulation of [^{35}S]-GTPγS binding to membrane preparations of C6-glial and HeLa cell lines expressing recombinant h5-HT$_{1A}$ receptors and their pK_i values for h5-HT$_{1A}$ receptors

	[^{35}S]-GTPγS binding response						
Cell type	*HeLa/h 5-HT$_{1A}$*		*C6-glial/h 5-HT$_{1A}$*		*C6-glial/h 5-HT$_{1A}$*		*[^{3}H]-8-OH-DPAT*
GDP (μM)	*30*		*0.3*		*30*		*binding HeLa/h 5-HT$_{1A}$*
	E_{max} (%)	*pEC_{50}*	*E_{max} (%)*	*pEC_{50}*	*E_{max} (%)*	*pEC_{50}*	*pK_i*
LY334370	89±2	7.15 (0.17)	83±2	7.24 (0.47)	94±4[a]	6.12 (0.24)	7.79 (0.05)
8-OH-DPAT	84±3	7.98 (0.19)	90±2	8.01 (0.18)	41±3[b]	7.41 (0.21)	8.88 (0.16)

Binding was performed with 500 pM [^{35}S]-GTPγS to membrane preparations of stably transfected C6-glial and HeLa cell lines as described in Methods. Mean E_{max} values ± s.e.mean of 4 to 6 independent experiments are expressed versus the stimulation obtained with 10 μM 5-HT. pEC_{50} values (presented as mean with 95% CL in parentheses) are defined as the concentration at which 50% of the maximal stimulation was obtained for each ligand. pK_i values were obtained with the HeLa membrane preparation. Statistical analysis was performed on the E_{max} values with the non-parametric Mann-Whitney U test. [a]$P<0.05$ *vs* LY334370 (C6-glial/h 5-HT$_{1A}$ 0.3 μM GDP), [b]$P<0.01$ *vs* 8-OH-DPAT (C6-glial/h 5-HT$_{1A}$, 0.3 μM GDP).

Abbreviations 5-CT, 5-carboxamidotryptamine; GR127935, (2′-methyl-4′-(5-methyl[1,2,4]oxadiazol-3-yl)biphenyl-4-carboxylic acid [4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]amide; [^{125}I]-GTI, 5-hydroxytryptamine-O-carboxyl-methyl-glycyl-[^{125}I]-tyrosinamide; 5-HT, 5-hydroxytryptamine; L694247, 2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-yl]ethanamine; LY334370, 5-(4-fluorobenzoyl)amino-3-(1-methylpiperidin-4-yl)-1H-indole fumarate; 8-OH-DPAT, 8-(hydroxy-2-(di-n-propylamino)tetralin; [^{125}I]-p-MPPI, [^{125}I]-4-(2′-methoxy-phenyl)-1-[2′-(n-2″-pyridinyl)-p-iodobenzamido]-ethyl-piperazine; SB224289, 1′-methyl-5-(2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carbonyl)-2,3,6,7-tetrahydrospiro[furo[2,3 f] indole-3,4′-piperidine]; WAY100635, N-[2-[4-(2-methoxyphenyl)1-piper-azinyl]ethyl]-N-(2-pyridinyl)cyclohexanecarboxamide

We thank Dr M. Perez for the synthesis of LY334370 and S. Cecco for expert secretarial assistance.

References

ADHAM, N., KAO, H-T., SCHECHTER, L.E., BARD, J., OLSEN, M., URQUHART, D., DURKIN, M., HARTIG, P.R., WEINSHANK, R.L. & BRANCHEK, T.A. (1993). Cloning of another human serotonin receptor (5-HT$_{1F}$): A fifth 5-HT$_1$ receptor subtype coupled to the inhibition of adenylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 408–412.

BEER, M.S., STANTON, J.A., HAWKINS, L.M. & MIDDLEMISS, D.N. (1993). 5-Carboxamidotryptamine-insensitive 5-HT$_1$-like receptors are concentrated in guinea-pig but not rat, claustrum. *Eur. J. Pharmacol.,* **236,** 167–169.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle-dye binding. *Anal. Biochem.,* **72,** 248–254.

DUPUIS, D.S., PALMIER, C., COLPAERT, F.C. & PAUWELS, P.J. (1998). Autoradiography of 5-HT$_{1A}$ receptor-activated G-proteins in guinea-pig brain sections by agonist-stimulated [^{35}S]-GTPγS binding. *J. Neurochem.,* (in press).

FARGIN, A., RAYMOND, J.R., REGAN, J.W., COTECCHIA, S., LEFKOWITZ, R.J. & CARON, M.G. (1989). Effector coupling mechanisms of the cloned 5-HT$_{1A}$ receptor. *J. Biol. Chem.,* **264,** 14848–14852.

GOZLAN, H., PONCHANT, M., DAVAL, G., VERGE, D., MENARD, F., VANHOVE, A., BEAUCOURT, J. P. & HAMON, M. (1988). ^{125}I-Bolton-hunter-8-methoxy-2-[N-propyl-N-propylamino]tetralin as a new selective radioligand of 5-HT$_{1A}$ sites in the rat brain. *In vitro* binding and autoradiographic studies. *J. Pharmacol. Exp. Ther.,* **224,** 751–759.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLACHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P.A. (1994). International union of pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol. Rev.,* **46,** 157–203.

JOHNSON, K.W., SCHAUSS, J.M., COHEN, M.L., AUDIA, J.E., KALDOR, S.W., FLAUGH, M.E., KRUSHINSKI, J.H., SHENCK, K.W.,KIEFER, A.D. Jr., NISSEN, J.S., DRESSMAN, B.A., ZGOMBICK, J.M., BRANCHEK, T.A., ADHAM, N. & PHEBUS, L.A. (1996). Inhibition of neurogenic protein extravasation in the dura via 5-ht$_{1F}$ receptor activation-implications to migraine therapy. *Abstract Soc. Neurosci.,* **22,** 528.10.

KUNG, M-P., FREDERICK, D., MU, M., ZHUANG, Z-P. & KUNG, H.F. (1995). 4-(2′-Methoxy-phenyl)-1-[2′-(n-2″-pyridinyl)-p-iodobenzamido]-ethyl-piperazine ([^{125}I]-p-MPPI) as a new selective radioligand of serotonin-1A sites in rat brain: *in vitro* binding and autoradiographic studies. *J. Pharmacol. Exp. Ther.,* **272,** 429–437.

LAPORTE, A.M., SCHECHTER, L.E., BOLANOS, F.J., VERGE, D., HAMON, M. & GOZLAN, H. (1991). [^{3}H]5-methyl-urapidil labels 5-HT$_{1A}$ receptors and α1 adrenoreceptors in the rat CNS. *In vitro* binding and autoradiographic studies. *Eur. J. Pharmacol.,* **198,** 59–67.

LUCAITES, V.L., KRUSHINSKI, J., SCHAUS, J.M., AUDIA, J.E., GEHLERT, D.R. & NELSON, D.L. (1996). Autoradiographic localization of the serotonin-1F (5-ht$_{1F}$) receptor in rat brain using [^{3}H]LY334370, a selective 5-ht$_{1F}$ receptor radioligand. *Abstract Soc. Neurosci.,* **22,** 528.14.

LUPARELLO, T.J. (1967). *Stereotaxic Atlas of the Forebrain of the Guinea-pig*. Basel (Switzerland), New York, S. Karger.

MENGOD,G., VILARO, M.T., RAURICH, A., LOPEZ-GIMENEZ, J.F., CORTES, R. & PALACIOS, J.M. (1996). 5-HT receptors in mammalian brain: receptor autoradiography and *in situ* hybridization studies of new ligands and newly identified receptors. *Histochem. J.,* **28,** 747–758.

OVERSHINER, C.D., ADHAM, N., ZGOMBICK, J.M., BRANCHEK, T.A., CALLIGARO, D.O., PHEBUS, L.A., ROUSH, M.E., JOHNSON, K.W., HEMRICK-LUECKE, S.K., FULLER, R.W., LUCAITES, V.L., WAINSCOTT, D.B., NELSON, D.L., WOLFF, M.C., BENVENGA, M.B., AUDIA, J.F., SCHAUS, J.M., KRUSHINSKI, J.H., KALDOR, S.W., DRESSMAN, B.A. & LEANDER, J.D. (1996). LY334370 is selective for the cloned 5-ht$_{1F}$ receptor. *Abstract Soc. Neurosci.,* **22,** 528.12.

PAUWELS, P.J., PALMIER, C., WURCH, T. & COLPAERT, F.C. (1996). Pharmacology of cloned human 5-HT$_{1D}$ receptor-mediated functional responses in stably transfected rat C6-glial cell lines: further evidence of differentiating human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **353,** 144–146.

PAUWELS, P.J., TARDIF, S., WURCH, T. & COLPAERT, F.C. (1997). Stimulated [^{35}S]GTPγS binding by 5-HT$_{1A}$ receptor agonists in recombinant cell lines: modulation of apparent efficacy by G-protein activation state. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **356,** 551–561.

PAUWELS, P.J., VAN GOMPEL, P.& LEYSEN, J.E. (1993). Activity of serotonin (5-HT) receptor agonists, partial agonists and antagonists at cloned human 5-HT$_{1A}$ receptors that are negatively coupled to adenylate cyclase in permanently transfected HeLa cells. *Biochem. Pharmacol.,* **45,** 375–383.

PHEBUS, L.A., JOHNSON, K.W., AUDIA, J.E., COHEN, M.L., DRESSMAN, B.A., FRITZ, J.E., KALDOR, S.W., KRUSHINSKI, J.H., SCHENCK, K.W., ZGOMBICK, J.M., BRANCHEK, T.A., ADHAM, N. & SCHAUSS, J.M. (1996). Characterization of LY334370, a potent and selective 5-ht$_{1F}$ receptor agonist, in the neurogenic dural inflammation model of migraine pain. *Abstract Soc. Neurosci.,* **22,** 528.11

SCOTT, E.E. & BRUINVELS, A.T. (1997). Visualisation of 5-HT receptors using [^{35}S]GTPS binding in rat brain. *Br. J. Pharmacol. Proc. Suppl.,* **120,** 297P.

SIJBESMA, H., SCHIPPER, J., CORNELISSEN, J.C.H.M., & DE KLOET, E.R. (1991). Species differences in the distribution of central 5-HT$_1$ binding sites: a comparative autoradiographic study between rat and guinea-pig. *Brain Res.,* **555,** 295–304.

SIM, L.J., SELLEY, D.E. & CHILDERS, S.R. (1995). *In vitro* autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[γ-[^{35}S]thio]-triphosphate binding. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7242–7246.

SIM, L.J., SELLEY, D.E., XIAO, R. & CHILDERS, S. R. (1996). Differences in G-protein activation by μ- and δ- opioid, and cannabinoid receptors in rat striatum. *Eur. J. Pharmacol.,* **307,** 97–105.

STANTON, J.A., HANDFORD, E.J. & BEER, M.S. (1997). *In vitro* visualization of 5-HT receptors in rat brain using [^{35}S]GTPγS binding. *Br. J. Pharmacol. Proc. Suppl.,* **120,** 121P.

STANTON, J.A., MIDDLEMISS, D.N. & BEER, M.S. (1996). Autoradiographic localization of 5-CT-insensitive 5-ht$_1$-like recognition sites in guinea pig and rat brain. *Neuropharmacology,* **35,** 223–229.

VERGE, D., DAVAL, G., MARCINKIEWICZ, M., PATEY, A., EL MESTIKAWY, S., GOZLAN, H. & HAMON, M. (1986). Quantitative autoradiography of multiple 5-HT$_1$ receptor subtypes in the brain of control or 5,7-dihydroxytryptamine-treated rats. *J. Neurosci.,* **6,** 3474–3482.

WAEBER, C. & MOSKOWITZ, M.A. (1995). [^{3}H]sumatriptan labels both 5-HT$_{1D}$ and 5-ht$_{1F}$ receptor binding sites in the guinea pig brain: an autoradiographic study. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **352,** 263–275.

WAEBER, C. & MOSKOWITZ, M.A. (1996). *In vitro* autoradiography study of guanylyl-5′-[γ-[^{35}S]thio]-triphosphate binding stimulated by 5-HT$_1$ agonists in guinea-pig. *Abstract Soc. Neurosci.,* **22,** 385.1.

WAINSCOTT, D.B., KRUSHINSKI, J.H., SCHAUSS, J.M., KALDOR, S.W., DRESSMAN, B.A., AUDIA, J.E., ADHAM, N. & NELSON, D.L. (1996). [^{3}H]LY334370, a selective radioligand for labeling the serotonin-1F (5-HT$_{1F}$) receptor. *Abstract Soc. Neurosci.,* **22,** 528.13.

WURCH, T., CHASTAGNIER, C., PALMIER, C., COLPAERT, F.C. & PAUWELS, P.J. (1996). A 413 bp region upstream the human 5-HT$_{1A}$ receptor gene is sufficient for its *in vitro* expression. *Neurosci. Res. Commun.,* **19,** 75–82.

(Received October 24, 1997
Revised January 22, 1998
Accepted February 16,1998)