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Binding profile of the novel 5-$HT_{1B/1D}$ receptor antagonist, [^{3}H]GR 125,743, in guinea-pig brain: a comparison with [^{3}H]5-carboxamidotryptamine

Valérie Audinot, Sonia Lochon, Adrian Newman-Tancredi, Gilbert Lavielle, Mark J. Millan *

Institut de Recherches Servier, Department of Psychopharmacology, 125 Chemin de Ronde, 78290 Croissy-sur-Seine, France

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Abstract

Native brain 5-$HT_{1B/1D}$ receptors were studied using the novel antagonist, [^{3}H]GR 125,743 (*N*-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)benzamide). In guinea-pig striatal membranes, [^{3}H]GR 125,743 displayed rapid association ($t_{1/2}$ = 4.5 min), high (90%) specific binding and high affinity (K_d = 0.29 nM), although B_{max} values (fmol/mg protein) varied according to brain region-striatum: 199; frontal cortex: 89; hippocampus: 79; cerebellum: 26. In frontal cortex, the B_{max} determined with [^{3}H]5-CT ([^{3}H]carboxamidotryptamine) was significantly higher (178; $P < 0.05$), suggesting that it also labels other binding sites. In striatal membranes, guanylylimidodiphosphate (GppNHp) inhibited [^{3}H]5-CT but not [^{3}H]GR 125,743 binding, suggesting that the latter has antagonist properties. Nevertheless, in competition binding experiments, the pK_i values obtained with [^{3}H]GR 125,743 and [^{3}H]5-CT for 20 serotonergic ligands, including L 694,247 (2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1*H*-indole-3-yl]ethylamine), GR 46,611 (3-[3-(2-dimethylamino-ethyl)-1*H*-indol-6-yl]-*N*-(4-methoxybenzyl)acrylamide), sumatriptan and alniditan, were highly correlated (r = 0.99). Ketanserin and ritanserin showed low affinity for [^{3}H]GR 125,743 binding to guinea-pig striatal sites (K_i = 12600 and 369 nM), suggesting that 5-HT_{1B} (rather than 5-HT_{1D}) receptors are predominantly labelled in this tissue. The present data indicate that [^{3}H]GR 125,743 is a useful tool for studying native 5-$HT_{1B/1D}$ receptors.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT_{1D} receptor; [^{3}H]GR 125,743; [^{3}H]5-CT; (Guinea pig)

1. Introduction

Recent years have seen the cloning of multiple serotonin (5-hydroxytryptamine, 5-HT) receptors which have been classified, on the basis of their primary structure and coupling to transduction mechanisms, into seven major classes, 5-HT_1 to 5-HT_7 (for review, see Boess and Martin, 1994). 5-$HT_{1B/1D}$ receptors, which belong to the 5-HT_1 receptor family, have attracted particular interest for several reasons. Firstly, they are located on peripheral sympathetic nerve terminals, cerebral blood vessels and primary afferent nocisponsive trigeminal neurones innervating the cerebral vasculature (Molderings et al., 1990; Bruinvels et al., 1992a; Hamel et al., 1993; Rebeck et al., 1994). This localization suggests that the activation of these populations of 5-$HT_{1B/1D}$ receptors by sumatriptan, a prototypical but non-selective 5-$HT_{1B/1D}$ receptor agonist, may, by either vascular and/or neurogenic mechanisms, contibute to its ability to relieve the pain of migraine (see Saxena and Ferrari, 1989; Moskowitz, 1992; Millan, 1995). Secondly, 5-$HT_{1B/1D}$ receptors are broadly distributed throughout the central nervous system of man, guinea pig and several other species, constituting the predominant 5-HT receptor subtype and being found in high concentrations in the dorsal horn of the spinal cord, basal ganglia, hippocampus and frontal cortex, consistent with a role in the modulation of nociception, motor behaviour and mood (Bruinvels et al., 1992b; Del Arco et al., 1993; Miller and Teitler, 1992; Lowther et al., 1992; Langlois et al., 1995; Middlemiss et al., 1996). Thirdly, 5-$HT_{1B/1D}$ receptors are found both postsynaptically to serotoninergic neurones and also as inhibitory autoreceptors, suggesting that they may be implicated in the pathogenenesis and treatment of anxiety, depression and other psychiatric disorders implicated in serotoninergic dysfunction (Waeber et al., 1990; Herrick-Davis et al., 1989). Although human and rat 5-HT_{1B} receptors (previously known as 5-$HT_{1D\alpha}$ and 5-HT_{1B} re-

* Corresponding author. Tel.: (33-1) 4118-2200; Fax: (33-1) 4118-2470.

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ceptors, respectively, Hartig et al., 1996) share a very high amino-acid sequence homology, a single amino-acid change in the transmembrane spanning regions confers distinct pharmacological profiles (Adham et al., 1992, 1994; Bruinvels et al., 1993, 1994; Hamblin and Metcalf, 1991; Weinshank et al., 1992; Metcalf et al., 1992; Oksenberg et al., 1992). This appears to afford high affinity at rat, but not human, 5-HT_{1B} sites for (−)-pindolol, (−)-propanolol, (−)-tertatolol and certain other alkylarylamine β-adrenoceptor antagonists (for a recent review and a discussion of nomenclature, see Hartig et al., 1996). 5-HT_{1B} receptors are more highly expressed than 5-HT_{1D} receptors in the central nervous system of man, guinea pig, rodents and other species (Bruinvels et al., 1994) and, unlike 5-HT_{1D} receptors, are localized on nerve terminals (Starkey and Skingle, 1994; Fink et al., 1995; Bühlen et al., 1996). In contrast, it has been suggested that a functional population of inhibitory 5-HT_{1D} autoreceptors may exist in the rat dorsal raphe nucleus (Piñeyro et al., 1995; Davidson and Stamford, 1995). Interestingly, whilst 5-HT_{1D} receptor mRNA has been found in human and guinea-pig trigeminal nuclei, in rat, 5-HT_{1B} receptor mRNA has been detected in this brain region (Bruinvels et al., 1992a; Rebeck et al., 1994). On the other hand, 5-HT_{1B} receptors have been detected in human cerebral vasculature (Hamel et al., 1993). To date, no highly selective ligands distinguishing 5-HT_{1D} and 5-HT_{1B} receptors have been described, although, in a preliminary study, SB 216641 (*N*-[3-(2-dimethylamino)ethoxy-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-(1,1′-biphenyl)-4-carboxamide) and BRL 15572 (1-phenyl-3-[4-(3-chlorophenyl)-piperizin-1-yl]phenylpropran-2-ol) are reported to bind preferentially to 5-HT_{1B} and 5-HT_{1D} receptors, respectively (Price et al., 1996). In addition, the protypical 5-HT_2 antagonists, ketanserin and ritanserin, possess 76- and 23-fold higher affinity, respectively, for 5-HT_{1D} compared with 5-HT_{1B} sites (Zgombik et al., 1995). In contrast, the novel selective 5-HT_{1D} antagonist, GR 127,935, shows 10-fold higher affinity for 5-HT_{1B} than 5-HT_{1D} sites (Clitherow et al., 1994; Skingle et al., 1996).

Previous radioligand binding studies of 5-$HT_{1B/1D}$ receptors have employed non-selective serotonergic agonists such as [^{3}H]5-HT or [^{3}H]5-CT, which necessitate the use of masking compounds to block binding to other sites, though [^{3}H]5-HT may still bind to 5-HT_{1E} sites (Leonhardt et al., 1989; Lowther et al., 1992; Miller and Teitler, 1992). Low concentrations of [^{3}H]5-CT do, in contrast, appear to selectively label 5-HT_{1D} sites in guinea-pig striatum and bovine caudate in the presence of masking agents (Mahle et al., 1991; Nowak et al., 1993). More recently, the novel and highly potent 5-HT_{1D} agonist [^{3}H]L 694,247 has also been presented as a radioligand. However, it displays only very low (36%) specific binding (Heald et al., 1994), and possesses significant (only 10-fold lower) affinity for 5-HT_{1A} receptors (Audinot et al., 1996; Newman-Tancredi et al., 1997b). Similarly, a further novel agonist, alniditan, which has been employed as a radioligand, is only 4-fold selective for 5-$HT_{1B/1D}$ receptors over 5-HT_{1A} receptors, while the prototypical 5-$HT_{1B/1D}$ agonist, sumatriptan, displays only modest affinity for these sites (Leysen et al., 1996; Waeber and Moskowitz, 1995). Finally, [^{125}I]-Serotonin-*O*-carboxymethyl-glycyl-tyrosinamide (GTI), which has significant selectivity for 5-$HT_{1B/1D}$ receptors, has been used in several studies (Boulenguez et al., 1992; Bruinvels et al., 1992b), but like the other ligands above, displays agonist activity and would be expected to label preferentially the G-protein coupled conformation of the receptor. Hence, there is a need for a radiolabelled antagonist of high affinity and marked selectivity at 5-$HT_{1B/1D}$ receptors, capable of labelling the whole receptor population. In this respect, the availability of a tritiated form of the antagonist GR 125,743, which is chemically-related to GR 127,935, is of particular interest since it possesses both high affinity and marked selectivity for 5-$HT_{1B/1D}$ receptors versus other 5-HT receptor subtypes as well as a diversity of other sites (Scopes et al., 1994; Audinot and Newman-Tancredi, unpublished observations). In vivo, GR 125,743 behaves as an antagonist in guinea pigs, reversing the hypothermia induced by the 5-$HT_{1B/1D}$ agonist GR 46,611 (Skingle et al., 1994; Hatcher et al., 1995; Monneyron et al., unpublished observations). The present study undertook a characterization of the binding profile of [^{3}H]GR 125,743 at native 5-$HT_{1B/1D}$ receptors in both guinea pig and bovine tissues. Its properties were directly compared to those of [^{3}H]5-CT.

2. Materials and methods

2.1. Membrane preparations

Frozen guinea-pig brain (Charles River, Saint-Aubin-les-Elbeuf, France) or bovine caudate (Cellubio, Courcelles-sous-Jouar, France) was homogenised using a Kinematica Polytron (setting 5, 15 s) in 20 volumes (weight/volume) of ice-cold Tris-HCl (50 mM, pH 7.7 at 22°C) containing $CaCl_2$ (4 mM) and ascorbic acid (0.1%). The homogenate was centrifuged (48 000 × *g*, 4°C, 25 min) and the pellet, resuspended in the same volume of buffer, was incubated at 37°C for 15 min to remove endogenous 5-HT. The suspension was recentrifuged (48 000 × *g*, 4°C, 25 min) and the pellet resuspended in 80 volumes of ice-cold buffer containing 10 μM pargyline ('assay buffer').

2.2. Radioligand binding

Radioligand binding assays utilised [^{3}H]5-CT (51.3 Ci/mmol, Du Pont NEN, Les Ulis France) and [^{3}H]GR 125,743 (70 Ci/mmol, Amersham, Les Ulis, France). Experiments were carried out in triplicate in a final volume of 500 μl of assay buffer. (+)-8-Hydroxy-2-

(di-*n*-propylamino)tetralin (8-OH-DPAT) and mesulergine (each at 100 nM, final concentration) were used to block [^{3}H]5-CT binding to 5-HT$_{1A}$ and 5-HT$_{2C}$ receptors, respectively (Nowak et al., 1993). 5-HT (10 μM) was used to define non-specific binding with both radioligands. In competition experiments, the concentrations of [^{3}H]5-CT and [^{3}H]GR 125,743 were 2.0 and 0.8 nM, respectively. Incubations, conducted for 60 min at 22°C, were terminated by rapid filtration using a Brandel Cell Harvester through Whatman GF/B filters pretreated with polyethylenimine 0.1%. The filters were rinsed three times with 5 ml ice-cold assay buffer. The addition of bovine serum albumin (0.1%) in the filtration buffer considerably lowered the non-specific binding of [^{3}H]GR 125,743 to filters. Experiments using GTP (guanosine triphosphate) or GppNHp (guanylylimidodiphosphate) were performed using a buffer containing Tris-HCl (50 mM, pH 7.4 at 22°C), $MgSO_4$ (10 mM), ascorbic acid (0.1%) and pargyline (10 μM), according to Herrick-Davis et al. (1988).

2.3. Data analysis

Data were analyzed by non-linear regression using the program PRISM (Graphpad Software Inc., San Diego, CA), to yield K_d (dissociation constant of the radioligand) and B_{max} (maximal binding density) values for saturation experiments, and IC_{50} (inhibitory concentration 50) and nH (pseudo-Hill coefficient) values for competition experiments. Inhibition constants (K_i) were calculated according to the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + L/K_d)$ where L is the concentration of radioligand. For correlation analysis, Pearson Product-Moment Correlation Coefficients were used.

2.4. Drugs

5-Hydroxytryptamine (5-HT) and nucleotides were purchased from Sigma (St. Quentin Fallavier, France), dihydroergotamine, (+)-8-hydroxy-2-(di-*n*-propylamino)tetralin ((+)8-OH-DPAT), (−)-propanolol and mesulergine from Research Biochemicals International (Natick, MA, USA), 2-[5-[3-(4-methyl-sulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1*H*-indole-3-yl]ethylamine (L 694,247) and 3-[3-(2-dimethylamino-ethyl)-1*H*-indol-6-yl]-*N*-(4-methoxybenzyl)acrylamide (GR 46,611) from Tocris Cookson (Bristol, UK) and metergoline from Farmitalia Carlo Erba (Milan, Italy). Ritanserin and ketanserin were obtained from Janssen (Beerse, Belgium), methiotepine from Hoffman-La Roche (Basel, Switzerland). Sumatriptan, 5-carboxyamidotryptamine (5-CT), *N*-methyl-2-[3-(1-methylpiperidin-4-yl)-1*H*-indol-5-yl] (GR 85,548), 5-fluoro-3-{3-[4-(5-methoxy-pyrimidin-4-yl)-piperazin-1-yl]-propyl}-1*H*-indole (BMS 181,101), alniditan, 2-[3-[(*R*,*S*)-2-(7-ethyl)-chromanylmethylamino]propylamino]]-1,4,5,6-tetrahydropyrimidine (a Janssen-patented alniditan analogue, internal Servier code: GLE 103), *N*-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)-benzamide (GR 125,743) and *N*-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-carboxamide (GR 127,935) were synthesised by Gilbert Lavielle (Servier, France).

3. Results

3.1. Binding kinetics of [^{3}H]GR 125,743 and [^{3}H]5-CT

The association of [^{3}H]GR 125,743 to guinea-pig striatal membranes was rapid and monophasic with an association half time ($t_{1/2}$) of 4.5 ± 0.64 min (Fig. 1A). Once equilibrium was attained, binding remained stable for at least 2 h (not shown). In contrast, dissociation isotherms

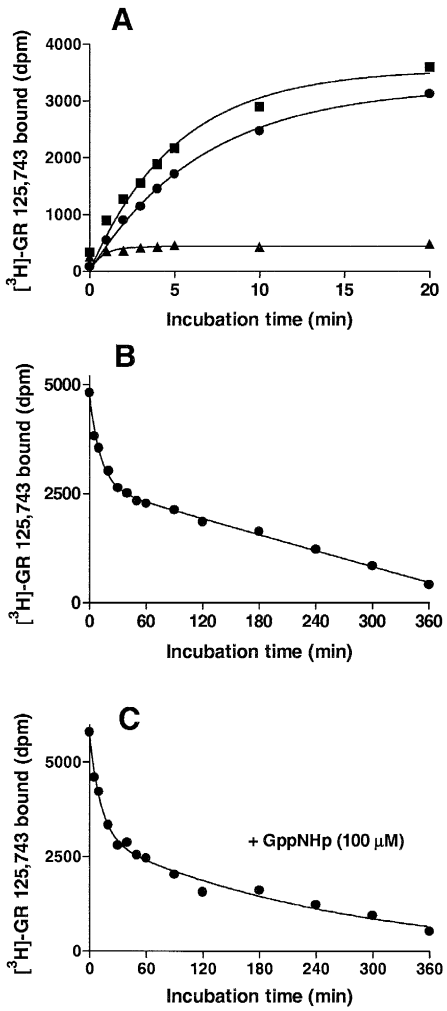

Fig. 1. Association (A) and dissociation (B,C) time-courses of [^{3}H]GR 125,743 (0.8 nM) binding to guinea-pig striatal membranes. For dissociation experiments of both radioligands, a 30 min incubation preceded addition of 5-HT (10 μM). When indicated (C), GppNHp (100 μM) was added in the incubation medium. Points shown are means of triplicate determinations from a single representative experiment, which was repeated on at least three independent occasions.

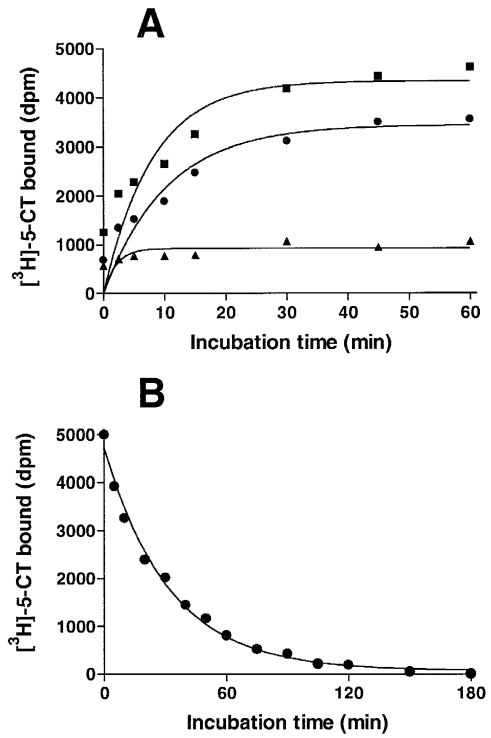

Fig. 2. Association (A) and dissociation (B) time-courses of [^{3}H]5-CT (2.0 nM) binding to guinea-pig striatal membranes. For dissociation experiments, a 30 min incubation preceded addition of 5-HT (10 μM). Points shown are means of triplicate determinations from a single representative experiment, which was repeated on at least three independent occasions.

were biphasic with a $t_{1/2}$ for the first component of 12 ± 2 min. After 1 hour of incubation, specific binding of [^{3}H]GR 125,743 was about 50% of its initial value, while the other 50% dissociated more slowly (Fig. 1B). This biphasic dissociation pattern was not altered in the presence of the non-hydrolysable GTP analogue, GppNHp (100 μM; Fig. 1C).

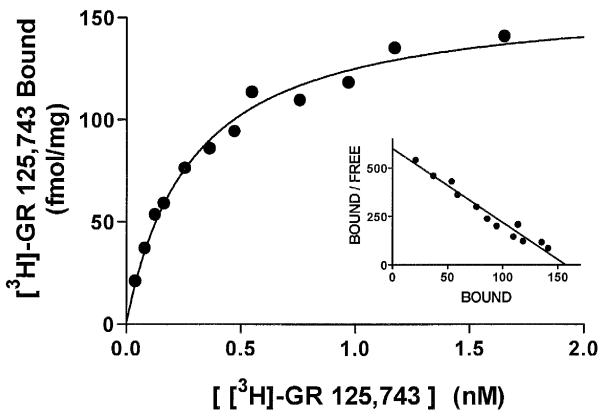

Fig. 3. Saturation binding isotherm of [^{3}H]GR 125,743 to guinea-pig striatal membranes. The scatchard analysis of the same data is shown in the inset. Points are means of triplicate determinations from a single representative experiment, which was repeated on at least three independent occasions.

Table 1
Saturation binding of [^{3}H]GR 125.743 and [^{3}H]5-CT to native brain membranes

Brain region	[^{3}H]GR 125,743		[^{3}H]5-CT	
	K_d	B_{max}	K_d	B_{max}
Guinea pig				
Striatum	0.29 ± 0.03	199 ± 16	1.23 ± 0.14	262 ± 23
Frontal cortex	0.24 ± 0.04	89 ± 1	1.60 ± 0.09	178 ± 10 [a]
Hippocampus	0.28 ± 0.01	79 ± 8	n.d.	n.d.
Cerebellum	0.29 ± 0.05	26 ± 2	0.48 ± 0.06	36 ± 2
Bovine				
Caudate	1.57 ± 0.21	179 ± 45	0.70 ± 0.08	187 ± 20

Values are means ± S.E.M. of at least three independent experiments performed in triplicate. n.d. = not determined.
[a] $P < 0.05$ in Student's t-test versus B_{max} for [^{3}H]GR 125,743.

Unlike [^{3}H]GR 125,743, both the association and dissociation isotherms of [^{3}H]5-CT were monophasic, resulting in $t_{1/2}$ values of 6.34 ± 0.85 min and 24 ± 4 min, respectively (Fig. 2A,B). The dissociation constant (K_d) for [^{3}H]5-CT calculated from these data was 1.13 nM.

3.2. Saturation experiments

Specific binding of [^{3}H]GR 125,743 to guinea-pig striatum (Fig. 3) and other brain regions was saturable and represented about 90% of total binding at concentrations

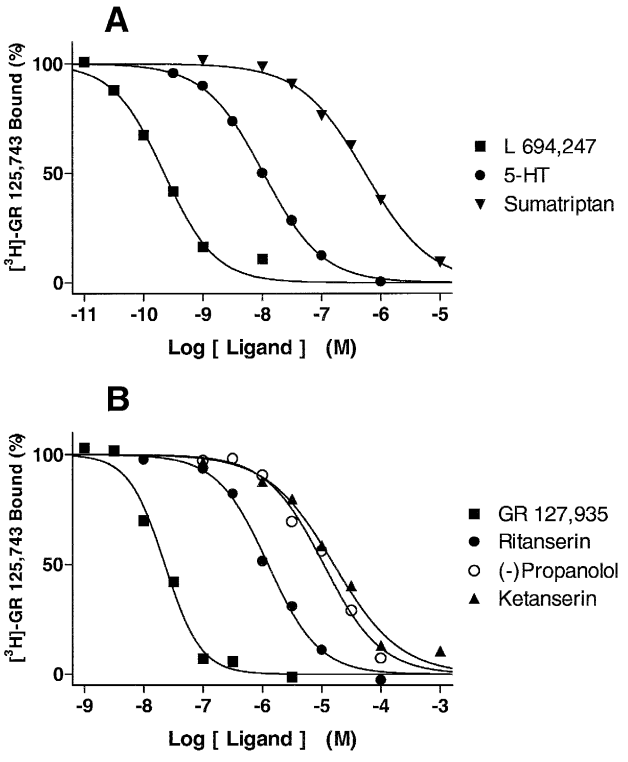

Fig. 4. Competition binding of [^{3}H]GR 125,743 binding with 5-HT$_{1D}$ agonists (A) and antagonists (B). Points shown are means of triplicate determinations from a single representative experiment, which was repeated on at least three independent occasions.

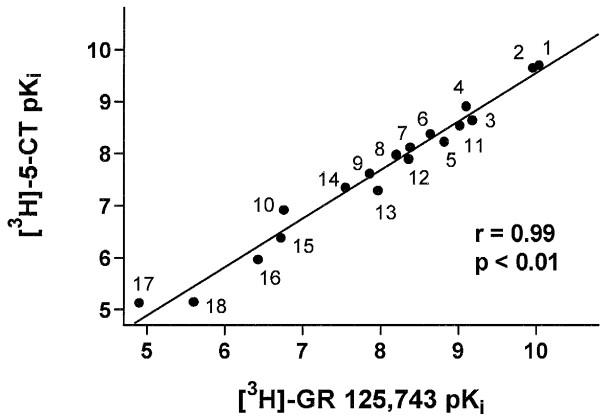

Fig. 5. Correlation analysis of pK_i values at guinea-pig striatal membranes determined with either [^{3}H]GR 125,743 or [^{3}H]5-CT as radioligand ($r = 0.99$, $P < 0.001$). Compounds are those listed in Table 2, with the exception of 8-OH-DPAT and mesulergine.

close to K_d. The K_d values were similar in the 4 guinea-pig brain regions examined (range 0.24–0.29 nM; Table 1). The K_d value of [^{3}H]5-CT was 4-fold higher than [^{3}H]GR 125,743 in striatum and frontal cortex and the maximal number of binding sites (B_{max}) was significantly higher with [^{3}H]5-CT in frontal cortex than with [^{3}H]GR 125,743 (Table 1). [^{3}H]GR 125,743 also labelled 5-HT$_{1B/1D}$ receptors in bovine caudate with a high affinity, which was, however, about 5-fold less than in guinea-pig striatum, and 2-fold lower than [^{3}H]5-CT in this tissue (Table 1). In bovine caudate, the number of labelled sites was similar with both radioligands.

3.3. Competition experiments

20 serotonergic ligands were tested for their affinity at guinea-pig striatal membranes using [^{3}H]GR 125,743 or [^{3}H]5-CT (Fig. 4, Table 2). The order of potency of the agonists tested was: L 694,247 > GR 46,611 > 5-CT > DHE > 5-HT > alniditan > BMS 181,101, GR85,548 > sumatriptan. The order of potency of the antagonists tested was: GR 125,743 > GR 127,935 > methiothepin > metergoline > methysergide > ritanserin ≫ ketanserin. The β-adrenoceptor/5-HT$_{1B}$ antagonist (−)-propanolol, the 5-HT$_{1A}$ agonist, 8-OH-DPAT, and the 5-HT$_{2C}$ antagonist, mesulergine, weakly inhibited [^{3}H]GR 125,743 binding. In all cases, the ligands tested inhibited binding of the two radioligands monophasically and binding isotherms were best fitted by a model which assumes the presence of a single class of binding sites. K_i values determined with [^{3}H]GR 125,743 were, on average, 1.8- and 2.6-fold lower than those determined with [^{3}H]5-CT for agonists and

Table 2
Ligand binding affinities (K_i) at guinea-pig striatal 5-HT$_{1B/1D}$ receptors

Competing ligand [a]	[^{3}H]GR 125,743		[^{3}H]5-CT	
	K_i (nM)	nH	K_i (nM)	nH
Agonists				
1. L 694,247	0.09 ± 0.02	1.08 ± 0.15	0.20 ± 0.06	0.83 ± 0.14
2. GR 46,611	0.11 ± 0.02	1.3 ± 0.2	0.22 ± 0.06	0.68 ± 0.21
3. GLE 103	0.66 ± 0.07	0.86 ± 0.06	2.3 ± 1.4	0.88 ± 0.04
4. 5-CT	0.79 ± 0.14	0.80 ± 0.04	0.72 ± 0.08	1.15 ± 0.16
5. Dihydroergotamine	1.5 ± 0.2	1.32 ± 0.13	5.9 ± 1.0	0.88 ± 0.10
6. 5-HT	2.3 ± 0.2	0.85 ± 0.03	2.6 ± 0.3	0.92 ± 0.03
7. Alniditan	4.2 ± 0.7	1.06 ± 0.17	7.5 ± 2.3	0.90 ± 0.09
8. BMS 181,101	6.3 ± 0.9	0.88 ± 0.06	10 ± 2	0.85 ± 0.05
9. GR 85,548	14 ± 4	1.03 ± 0.23	24 ± 4	0.85 ± 0.05
10. Sumatriptan	175 ± 66	0.72 ± 0.07	119 ± 15	0.97 ± 0.07
Antagonists				
11. GR 125,743	0.94 ± 0.14	1.28 ± 0.18	2.9 ± 0.2	0.79 ± 0.06
12. GR 127,935	4.3 ± 1.2	1.20 ± 0.13	13 ± 3	0.76 ± 0.04
13. Methiotepine	11 ± 1	0.94 ± 0.05	51 ± 9	0.54 ± 0.01
14. Metergoline	28 ± 2	1.14 ± 0.03	44 ± 4	1.13 ± 0.06
15. Methysergide	188 ± 28	1.03 ± 0.16	420 ± 69	0.94 ± 0.02
16. Ritanserin	369 ± 55	1.14 ± 0.12	1063 ± 119	0.87 ± 0.09
17. Ketanserin	12 609 ± 4308	0.84 ± 0.14	7464 ± 3295	0.61 ± 0.05
Miscellaneous				
18. (−)Propanolol	2744 ± 174	0.96 ± 0.04	7643 ± 1303	0.84 ± 0.06
8-OH-DPAT	523 ± 211	0.73 ± 0.05	n.d.	n.d.
Mesulergine	9105 ± 2706	1.01 ± 0.04	n.d.	n.d.

Inhibition constants (K_i) and Hill coefficients (nH) for the inhibition of [^{3}H]GR 125,743 and [^{3}H]5-CT binding to guinea-pig striatal membranes. Binding isotherms were best fitted by a single site model. Values are means ± S.E.M. of at least three independent experiments performed in triplicate. n.d. = not determined.

[a] Compounds are numbered for cross-referencing to Fig. 5.

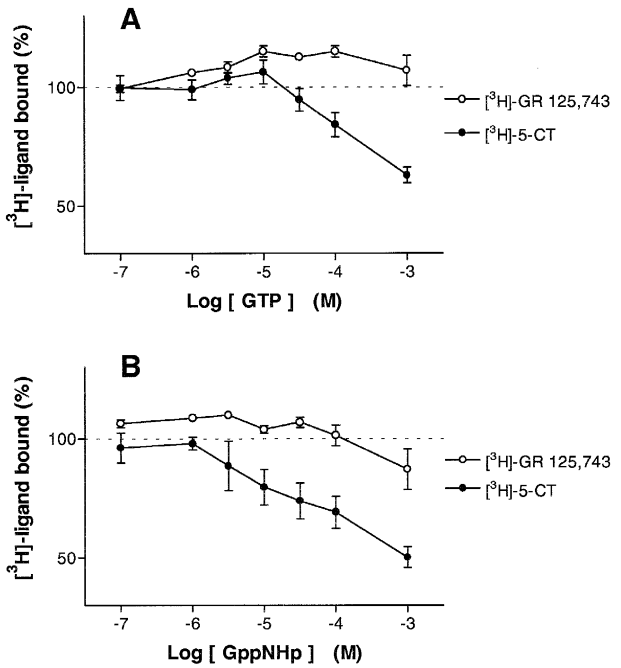

Fig. 6. Modulation by guanine nucleotides of [^{3}H]GR 125,743 and [^{3}H]5-CT binding. (A) Effect of GTP; (B) effect of GppNHp. Points shown are means of four independent experiments, each performed in triplicate.

antagonists, respectively. In both cases, this difference was statistically significant ($P < 0.05$, one sample *t*-test, theoretical ratio of $K_i = 1$). Nevertheless, the pK_i values determined with the two radioligands were highly correlated ($r = 0.99$, $P < 0.001$, Fig. 5),

3.4. Guanine nucleotide modulation

[^{3}H]5-CT binding was concentration dependently inhibited by GTP and GppNHp whereas [^{3}H]GR 125,743 binding was not affected by these nucleotides (Fig. 6). No significant effects on [^{3}H]5-CT binding were observed with concentrations up to 10 mM of GMP, GDP, GppNHp or ATP (data not shown).

4. Discussion

4.1. [^{3}H]GR 125,743: a potent and selective antagonist at 5-HT$_{1B/1D}$ receptors

Scopes et al. (1994) first reported that the novel 5-HT$_{1B/1D}$ ligand, GR 125,743 behaves as a potent and selective antagonist at 5-HT$_{1D}$ receptors with 'ca. 100-fold selectivity over 5-HT receptor subtypes' and suggested that it may be a useful tool to investigate the function of 5-HT$_{1B/1D}$ receptors. Indeed, in our hands, GR 125,743 was highly selective for 5-HT$_{1B/1D}$ receptors over a dozen other receptor subtypes, including adrenoceptors, serotonin and dopamine receptors (Audinot and Newman-Tancredi, unpublished observations). GR 125,743 also has higher selectivity for 5-HT$_{1B/1D}$ over 5-HT$_{1A}$ receptors than the structurally related biarylanilide antagonist, GR 127,935. The latter shows marked affinity at human recombinant 5-HT$_{1A}$ receptors transfected into a Chinese Hamster Ovary cell line (10-fold difference to 5-HT$_{1D}$ sites, Audinot et al., 1996; Newman-Tancredi et al., 1997b), whereas GR 125,743 is 56-fold selective for 5-HT$_{1D}$ sites ($K_i = 53.1$ nM at human 5-HT$_{1A}$, versus 0.94 nM at guinea-pig 5-HT$_{1B}$ receptors; Newman-Tancredi et al., 1997b).

In striatal membranes, saturation experiments with [^{3}H]GR 125,743 yielded subnanomolar K_d values, 4-fold lower than that of [^{3}H]5-CT (0.29 vs. 1.23 nM) demonstrating the high potency of this radioligand. [^{3}H]GR 125,743 also labelled 5-HT$_{1B/1D}$ receptors in guinea-pig frontal cortex, hippocampus and cerebellum with K_d values close to that found in the striatum, suggesting that a similar population of 5-HT$_{1B/1D}$ receptors was labelled in these four brain regions (Bruinvels et al., 1993). Further, the B_{max} values obtained across the various tissues with [^{3}H]GR 125,743 (striatum > frontal cortex = hippocampus ≫ cerebellum) correspond to previous studies of the relative densities of 5-HT$_{1B/1D}$ sites and their corresponding mRNA in these brain regions (Del Arco et al., 1993; Miller and Teitler, 1992; Lowther et al., 1992; Langlois et al., 1995). The density of sites labelled by [^{3}H]5-CT was generally higher, this difference attaining statistical significance in frontal cortex (Table 1) and suggesting that [^{3}H]GR 125,743 selectively labels 5-HT$_{1D}$ receptors whereas [^{3}H]5-CT may not. However, the affinity of 5-CT for these (putative) additional sites appears to be close to its affinity at 5-HT$_{1B/1D}$ sites since saturation experiments with [^{3}H]5-CT were best fitted by a one-site analysis both in striatum and frontal cortex. It is of interest that 5-CT exhibits almost equally high affinity for 5-HT$_7$ and 5-HT$_{1D}$ receptors (Tsou et al., 1994) and, in the presence of compounds masking 5-HT$_{1A}$, 5-HT$_{2C}$ and 5-HT$_{1D}$ receptors, [^{3}H]5-CT can be used to label 5-HT$_7$ sites in guinea pig and rat brain (To et al., 1995; Gustafson et al., 1996). Hence the higher B_{max} value observed with [^{3}H]5-CT versus [^{3}H]GR 125,743 could represent binding at 5-HT$_7$ receptors. Accordingly, the B_{max} values for [^{3}H]5-CT and [^{3}H]GR 125,743 were similar in the cerebellum, a brain region expressing very few 5-HT$_7$ receptors (To et al., 1995). Nevertheless, arguing against a major contribution of 5-HT$_7$ receptors, is the presence in the incubation medium of 8-OH-DPAT and mesulergine at 100 nM (see Section 2), concentrations close to their K_i values at 5-HT$_7$ receptors (Tsou et al., 1994) which would be expected to occupy a large proportion of these sites. Further, clozapine, a potent 5-HT$_7$ receptor ligand (K_i = 6.3 nM, Roth et al., 1994) showed only low affinity for 5-HT$_{1D}$ sites labelled by [^{3}H]5-CT in frontal cortex (5890 nM, unpublished result). Thus, although 5-HT$_7$ receptors may be involved, the identity of this additional site recognised by [^{3}H]5-CT remains to be further elucidated. It is

possible that actions at this additional site contribute to the shallow [^{3}H]5-CT competition binding isotherms observed in guinea-pig striatum with ketanserin, methiotepin and GR 46,611. Irrespective of the nature of these sites, these data highlight the usefulness of [^{3}H]GR 125,743, rather than [^{3}H]5-CT, to selectively label 5-HT$_{1B/1D}$ receptors. Interestingly, [^{3}H]GR 125,743 also bound potently to 5-HT$_{1B/1D}$ receptors in bovine caudate, and the density of labelled sites were similar with [^{3}H]5-CT and [^{3}H]GR 125,743, suggesting that there was no difference in the binding sites recognised in this tissue. Indicative of the more general potential use of [^{3}H]GR 125,743, results obtained in rat frontal cortex showed that this radioligand also potently and selectively labels 5-HT$_{1B}$ sites in this species (Audinot, unpublished observations). No published information is available concerning the interaction of GR 125,743 with subtypes of 5-HT$_{1B}$ versus 5-HT$_{1D}$ receptors. However preliminary results indicate that GR 125,743 is equipotent at recombinant human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors (K_i values vs [^{3}H]GR 125,743 are, respectively, 1.9 and 1.1 nM, unpublished observations). The related biarylanilide, GR 127,935 posseses a 10-fold preference for cloned human 5-HT$_{1B}$ versus 5-HT$_{1D}$ receptor sutbypes (Skingle et al., 1996), and its high affinity at the sites labelled by [^{3}H]GR 125,743 and [^{3}H]5-CT in the guinea-pig striatum are consistent with these being of the 5-HT$_{1B}$ subtype. Further, ritanserin and ketanserin, which display markedly higher affinity at recombinant human 5-HT$_{1D}$ versus 5-HT$_{1B}$ receptors (Zgombik et al., 1995), only show weak affinity at native guinea-pig 5-HT$_{1D}$ receptors. These findings strongly suggest that GR 125,743 is labelling primarily 5-HT$_{1B}$ sites in guinea-pig striatum, in accordance with the preponderant expression of the 5-HT$_{1B}$ receptor subtype in rat brain (Bruinvels et al., 1993). However, as mentioned above, this radioligand may also label a minority of 5-HT$_{1D}$ sites. An analysis of binding isotherms with a greater number of concentrations of ritanserin and GR 127,935 would be necessary to define the proportion of these two populations of binding sites in native tissues.

4.2. Inhibition of [^{3}H]5-CT but not [^{3}H]GR 125,743 binding by guanine nucleotides

The present in vitro binding data support previous reports of the in vivo antagonist properties of GR 125,743 (Scopes et al., 1994; Hatcher et al., 1995). Indeed, whereas the binding of [^{3}H]5-CT to guinea-pig 5-HT$_{1D}$ sites was concentration dependently decreased by GTP (Herrick-Davis et al., 1988; Nowak et al., 1993; present study), [^{3}H]GR 125,743 binding was not modified by GTP or its non-hydrolysable analogue, GppNHp. These guanine nucleotides can induce dissociation of the G-protein from the coupled receptor and, therefore, the observed inhibition of binding is an indication that the radioligand binds with higher affinity to G-protein-coupled receptors, behaviour which is typical of agonists. In comparison, the binding of *inverse* agonists is increased by GTP (see for example Sundaram et al., 1993), whereas insensitivity to GTP/GppNHp is evidence of 'neutral' antagonist properties, as is the case here for [^{3}H]GR 125,743. It would therefore be predicted that this radioligand, unlike [^{3}H]5-CT, would label both G-protein-coupled and -uncoupled receptors with similar affinity, enabling the investigation of the total receptor population. The observation that both [^{3}H]5-CT and [^{3}H]GR 125,743 label similar numbers of binding sites in guinea-pig striatum and cerebellum and in bovine caudate, suggests that 5-HT$_{1B/1D}$ receptors in these tissues may all exist in a G-protein-coupled state accessible to both agonist and antagonist radioligands. This is consistent with an absence of receptor reserve in these terminal brain regions, although possible receptor reserve at cell body autoreceptors, as is known to exist for other receptor subtypes, such as 5-HT$_{1A}$ (Meller et al., 1990), remains to be investigated. Nevertheless, in autoradiographic experiments, [^{3}H]GR 125,743 reportedly labels considerably higher numbers of binding sites in rat brain slices than agonist radioligands, although this may be due to differences in buffer/incubation conditions (Mengod et al., 1996). In any case, GR 125,743 should be tested in models of 5-HT$_{1B/1D}$ receptor-coupled transduction mechanisms at both native and cloned receptors in order to further characterize its antagonist properties at a molecular level. Its structural analogue, GR 127,935 shows weak agonist properties in transfected cells, whereas in vivo both GR 125,743 and GR 127,935 have been described as 'silent' antagonists in the guinea-pig model of hypothermia (Walsh et al., 1995; Pauwels and Palmier, 1995; Newman-Tancredi et al., 1997a; Skingle et al., 1994; Hatcher et al., 1995). Although the precise degree of intrinsic activity of these ligands remains to be clarified, these differences may be related to variations in receptor reserve and transduction machinery between recombinant and native tissues.

4.3. Binding kinetics of [^{3}H]GR 125,743

An analysis of the association/dissociation kinetics of [^{3}H]GR 125,743 revealed a biphasic effect on the dissociation time course. The precise reasons underlying this remain to be determined, but several points may be noted. Firstly, it might be argued that [^{3}H]GR 125,743 recognises both the G-protein-coupled and -uncoupled states of the receptors, and that these correspond to the two phases seen. However, this is unlikely in view of the fact that the non-hydrolysable GTP analogue, GppNHp, did not inhibit [^{3}H]GR 125,743 binding (as discussed above) or modify the pattern of dissociation, confirming the in vitro antagonistic properties of the radioligand. Secondly, biphasic dissociation was not a specific characteristic of guinea-pig striatal 5-HT$_{1B/1D}$ receptors, since, under the same conditions, [^{3}H]5-CT dissociated monophasically. Thirdly, the complex dissociation pattern of [^{3}H]GR 125,743 appears to be an intrinsic property of this ligand, since it was also observed for the binding of [^{3}H]GR 125,743 to the frontal

cortex of the rat: that is, in a different brain region expressing the rat 5-HT_{1B} receptor homologue of guinea-pig 5-HT_{1B} receptors (unpublished observations). These data suggest that a ligand-dependent conformational change of the receptor may be taking place resulting in a slower dissociation rate. Such ligand-dependent effects may also be involved in receptor 'trafficking' (Kenakin, 1995; Kenakin, 1996) and in differentiating agonists/partial agonist effects. Interestingly, in vitro and in vivo data indicate that GR 127,935, the analogue of GR 125,743, may dissociate slowly, and possibly incompletely, from 5-HT_{1D} receptors (Skingle et al., 1996).

4.4. Competition binding profiles of [^{3}H]GR 125,743 and [^{3}H]5-CT

Competition experiments in guinea-pig striatum gave K_i values that were generally about two fold lower with [^{3}H]GR 125,743 than with [^{3}H]5-CT. The reasons for this are unclear but this difference, which attained statistical significance ($P < 0.05$; see Section 3), may be due to induction/stabilisation of the receptor in a high affinity state by [^{3}H]GR 125,743, as discussed above in relation to its biphasic dissociation pattern. Nevertheless, the order of potency of the competing ligands was the same for both radioligands and respective pK_i values were highly correlated ($r = 0.99$) suggesting that, at concentrations used for competition experiments, they recognise the same population of 5-$HT_{1B/1D}$ receptors. All ligands, including agonists, yielded monophasic isotherms for competition with [^{3}H]GR 125,743 indicating that a single population of sites was recognized. These are likely to represent the high-affinity state of the receptor since the K_i values were close to those determined with [^{3}H]5-CT which, as discussed above, preferentially labels receptors which are coupled to G-proteins. Amongst the agonists tested, L 694,247 displayed exceptional potency, in agreement with results obtained in previous binding studies of cloned and native 5-HT_{1D} sites and several in vitro models of coupling to second messenger systems (Beer et al., 1993; Heald et al., 1994). GR 46,611 was also a highly potent agonist, and is active in several in vivo models (Skingle et al., 1994, 1996). The overall order of potency of the agonists tested is in global agreement with previous reports (Hoyer and Shoeffter, 1991; Beer et al., 1993; Nowak et al., 1993; Leysen et al., 1996; Clitherow et al., 1994; Adham et al., 1992). Unlike previous studies, however, the present investigation tested all the compounds on the same tissue under identical conditions enabling direct comparisons of affinities.

4.5. Conclusions

The present data suggest that the novel, selective, 5-$HT_{1B/1D}$ antagonist [^{3}H]GR 125,743 is a useful tool for the radiolabelling of native 5-$HT_{1B/1D}$ receptors in several species. Its antagonist properties and selectivity present particularly important features compared with 5-CT and other agonist radioligands. It will, nevertheless, be important to establish the reasons underlying its distinctive biphasic pattern of dissociation, to identify and quantify the nature of the 5-$HT_{1B/1D}$ sites recognised (G-protein-coupled or uncoupled) and to further characterize its putative antagonist properties at both native and heterologously expressed recombinant 5-$HT_{1B/1D}$ receptors subtypes.

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