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European Journal of Pharmacology 386 (1999) 97–103

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Correlation between low/high affinity ratios for 5-HT_{1A} receptors and intrinsic activity

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Received 5 July 1999; received in revised form 14 October 1999; accepted 19 October 1999

Abstract

G protein-coupled receptors exist in G protein-coupled and -uncoupled forms that exhibit high and low affinity for agonists, respectively. Consequently, affinity differences of a compound for the high vs. the low affinity state of a receptor have been used to estimate its intrinsic activity at that receptor. We examined the affinity of a series of compounds for 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor sites labeled with 0.2 nM [^{3}H](±)-8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) (high affinity), or with 0.25 nM [^{3}H]4-(2′-methoxy-)-phenyl-1-[2′-(*N*-2″-pyridyl)-*p*-fluorobenzamido]ethyl-piperazine ([^{3}H]*p*-MPPF) in the presence of 100 μM guanylylimidodiphosphate (Gpp(NH)p) (low affinity) in rat hippocampal membranes. For a variety of 5-HT_{1A} receptor ligands, the low/high affinity ratio (ranging from 110 for 5-HT to 0.12 for spiperone) was in good agreement with their reported intrinsic activity. Positive rank correlations were found between low/high affinity ratios and intrinsic activities (E_{max} values) reported in the literature. The high efficacy 5-HT_{1A} receptor agonists, 1[2-(4-fluorobenzoylamino)ethyl]-4-(7-methoxynaphtyl)piperazine (S-14506) and dihydroergotamine, however, had similar, high affinity for both G protein-coupled and -uncoupled forms of the receptor. The Hill coefficients for both compounds were markedly higher than 1.0, suggesting that positive cooperativity could be responsible for the unexpected results. The 5-HT_{1A} receptor agonist activity of dihydroergotamine and S-14506, assessed by measuring the inhibition of forskolin-stimulated cAMP accumulation, was blocked completely by pertussis toxin, reinforcing the suggested involvement of an inhibitory G protein in their effects. Taken together, the results suggest that, although the low/high affinity ratio of a ligand for 5-HT_{1A} receptors generally covaries with its intrinsic activity, dihydroergotamine and S-14506 may interact with 5-HT_{1A} receptors in a manner different from that of other 5-HT_{1A} receptor agonists. Their effects, however, appear to be G_i protein-dependent.

Keywords: Affinity; Intrinsic activity; Efficacy; Gpp(NH)p shift; 5-HT_{1A} receptor ligand; S-14506; Dihydroergotamine

1. Introduction

G protein-coupled receptors can exist in different states. These states have different affinity for agonists, but similar affinity for antagonists. The difference in affinity of a compound for these states can give an indication of its intrinsic activity (see Birdsall and Lazareno, 1997). Agonist radioligands label the high affinity, G protein-coupled, state of the receptor, but dissociation of the G protein–receptor complex results in free receptor subunits with low affinity for agonists, which can no longer be labeled by agonist radioligands (Emerit et al., 1990). In contrast, the binding of antagonist radioligands to G protein-coupled receptors is known to be independent of this coupling (Kobilka, 1992); thus, antagonist radioligands label both affinity states with high affinity. Biphasic inhibition curves can be obtained with agonists, but only when the high and low affinities are distinct enough to be resolved; thus, it is often difficult to dissociate the two components of agonist ligand binding under these conditions. Nonhydrolysable GTP analogues such as guanylylimidodiphosphate (Gpp(NH)p) cause the receptor/G protein complex to dissociate, and shift the receptor conformation to the low affinity state. Under these conditions, the low affinity component of the binding of an agonist can be measured more accurately using an antagonist radioligand. Such conditions have been used by Lahti et al. (1992) to characterize dopamine D_2 receptor ligands.

[^{3}H](±)-8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) has been the prototypical agonist radioligand for 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor binding for

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00738-4

more than 15 years (Gozlan et al., 1983), but it is only in the last few years that selective antagonists for these receptors have been identified. One such radiolabeled compound, [^{3}H]4-(2′-methoxy-)-phenyl-1-[2′-(*N*-2″-pyridyl)-*p*-fluorobenzamido]ethyl-piperazine ([^{3}H]*p*-MPPF), is now commercially available (Kung et al., 1996).

The present work was undertaken to examine the high and low affinities of various 5-HT$_{1A}$ receptor ligands for 5-HT$_{1A}$ receptors in rat hippocampus. The high affinity component was assessed in competition experiments with [^{3}H]8-OH-DPAT, and the low affinity component by using [^{3}H]*p*-MPPF in the presence of Gpp(NH)p. For some compounds with high intrinsic activity, the inhibition of [^{3}H]*p*-MPPF was also studied in the absence of Gpp(NH)p. For various 5-HT$_{1A}$ receptor ligands, the ratio of K_i values for low/high affinity states was estimated and compared with intrinsic activity reported in the literature (Pauwels et al., 1997; Koek et al., 1998; Newman-Tancredi et al., 1998). In general, affinity ratios correlated positively with intrinsic activity. 1[2-(4-Fluorobenzoylamino)ethyl]-4-(7-methoxynaphtyl)piperazine (S-14506) (Colpaert et al., 1992) and dihydroergotamine, however, although having high intrinsic activity (Newman-Tancredi et al., 1998; Pauwels et al., 1998), had low/high affinity ratios less than one. To verify the involvement of a G_i protein in the agonist effects of these compounds, their ability to inhibit forskolin-stimulated cAMP accumulation in HeLa cells permanently expressing 5-HT$_{1A}$ receptors (HA7 cells) was examined in the presence and in the absence of pertussis toxin.

2. Materials and methods

2.1. Radioligand binding at 5-HT$_{1A}$ receptors

2.1.1. Membrane preparations

Frozen brains of male Sprague–Dawley rats [Ico: OFA SD (I.O.P.S. Caw); Iffa Credo, France], weighing 180–200 g, were used in all experiments and were stored at −70°C before use in binding assays.

5-HT$_{1A}$ receptor binding experiments were carried out in membrane preparations from rat hippocampus. Frozen brains were thawed, the hippocampi were dissected and homogenized in 20 volumes of ice-cold Tris–HCl (50 mM, pH 7.4 at 25°C). The homogenate was centrifuged at 39 000 × *g* for 10 min, the pellet was resuspended in the same volume of buffer and was recentrifuged as before. Following a further resuspension, the tissue was incubated for 10 min at 37°C and centrifuged again. The final pellet was suspended in the same buffer. The final tissue concentration was 3 mg/assay tube.

2.1.2. Binding experiments

Experiments were carried out essentially as described by Hall et al. (1985) for [^{3}H]8-OH-DPAT binding and by Thielen et al. (1996) for [^{3}H]*p*-MPPF binding. For saturation experiments, the incubation medium consisted of 0.1 ml of the different concentrations of either [^{3}H]8-OH-DPAT (ranging from 0.125 to 16 nM) or [^{3}H]*p*-MPPF (ranging from 0.05 to 6.4 nM), 0.1 ml of buffer or 5-HT (10 μM to define non-specific binding), and 0.8 ml of membrane preparation (with or without 100 μM Gpp(NH)p for [^{3}H]*p*-MPPF binding). For competition experiments, the incubation medium consisted of 0.1 ml of [^{3}H]8-OH-DPAT (0.2 nM) or [^{3}H]*p*-MPPF (0.25 nM), 0.1 ml of the different concentrations of the test compound (15 concentrations), and 0.8 ml of membrane preparation (containing 100 μM Gpp(NH)p for [^{3}H]*p*-MPPF binding, unless stated otherwise). The assay tubes were incubated for 30 min at 23°C for [^{3}H]8-OH-DPAT binding and at 37°C for [^{3}H]*p*-MPPF binding. The reaction was terminated by rapid filtration, using a Brandel harvester, through GF/B fiber filters with two 4-ml washes of Tris buffer. The radioactivity retained on the filters was measured by scintillation spectroscopy in 4 ml of scintillation fluid (Emulsifier safe, Packard). All experiments were performed in triplicate.

Results were analyzed using the non-linear curve fitting program EBDA/LIGAND (Biosoft, Cambrige, UK) (McPherson, 1985). The dissociation constant (K_d) and the total number of binding sites (B_{max}) for each radioligand were estimated from saturation experiments. Results from competition experiments are expressed as mean pK_i values (± S.E.M.) based on three determinations, and the mean K_i low/K_i high ratio and its 95% confidence limits were calculated for each compound. In addition to pK_i values, slope factors (equivalent to the Hill coefficient) were also calculated.

The relationships between the low/high affinity ratios observed here and the intrinsic activities reported in the literature were examined by calculating Spearman's rank order correlations (r_s) by means of the program SigmaStat (SPSS, Chicago). Rank correlations were used because intrinsic activities were measured as relative maximal responses, which cannot be directly equated to the molecular properties of agonism except in a sense of rank order (i.e., the agonist that produces the larger maximal response possesses the greater efficacy) (Kenakin, 1993).

2.2. Measurement of cyclic AMP accumulation in HA7 cells

The HeLa cell line permanently transfected with the human 5-HT$_{1A}$ receptor gene and permanently expressing the 5-HT$_{1A}$ receptor protein, 500 fmol/mg protein, (HA7) as described by Fargin et al. (1989), was commercially obtained from Duke University, Durham, NC, USA. HA7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal calf serum, gentamicin (100 μg/ml), geneticin (G418) (400 μg/ml) in 5% CO_2 at 37°C in a water-saturated atmosphere. The cells were plated in 6-well culture plates and used for

Table 1
Binding parameters of [^{3}H]8-OH-DPAT or [^{3}H] *p*-MPPF (in the absence or presence of 100 μM Gpp(NH)p) to membrane preparations from rat hippocampus (results are means ± S.E.M. of three determinations)

Ligand	K_d (nM)	B_{max} (fmol/mg wet weight)
[^{3}H]8-OH-DPAT	0.49 ± 0.02	16.3 ± 1.5
[^{3}H] *p*-MPPF	0.45 ± 0.01	35.8 ± 2.0
[^{3}H] *p*-MPPF + Gpp(NH)p	0.56 ± 0.07	34.1 ± 2.0

experimentation at confluency of 80–90%. Culturing medium was replaced by DMEM supplemented with 10% fetal calf serum and antibiotics, containing or not 200 ng/ml pertussis toxin, 16 h before agonist treatment.

After pertussis toxin treatment, cells were washed twice with phosphate buffered saline and preincubated with DMEM, 10 mM HEPES for 10 min at room temperature. S-14506 and dihydroergotamine, at concentrations varying from 0.1 nM to 100 μM were then added in DMEM, 10 mM HEPES, 100 μM forskolin, and 100 μM 3-isobutyl-1-methylxanthine to the cells. At the end of the treatment (10 min, room temperature), the reaction was stopped by aspiration of the medium and addition of 0.1 N HCl. cAMP content was measured by radioimmunoassay using a commercially available kit (Dupont NEN: NEK-033). Basal cAMP levels were 10 ± 0.9 pmol/well ($n = 8$).

Concentration–effect relationships are expressed as −log [M] of the test compound vs. the cAMP content expressed as a percentage of forskolin-stimulated cAMP. Each concentration–response experiment was performed in triplicate. IC_{50} values were estimated using the mean values of three separate experiments, by means of non-linear regression (Sigmoidal model, GraphPad Prism).

2.3. Chemicals

[^{3}H]8-OH-DPAT (TRK 850; 160–240 Ci/mmol) was purchased from Amersham France (Les Ulis, France), [^{3}H] *p*-MPPF (NET 1109; 60–87 Ci/mmol) from NEN Life Science Products (Paris, France). 5-HT creatinine sulphate, (−)-8-OH-DPAT hydrobromide, (+)-8-OH-DPAT hydrobromide, (±)-8-OH-DPAT hydrobromide, 5-carboxamidotryptamine maleate (5-CT), buspirone hydrochloride, (−)-pindolol, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine (NAN-190) hydrobromide, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro-[4,5]decane-7,9-dione (BMY 7378) dihydrochloride, clozapine, dihydroergotamine mesylate, forskolin, spiperone hydrochloride, and spiroxatrine were purchased from Sigma-RBI (Saint Quentin Fallavier, France); 2-[5-[3-(4-methylsulphonylamino)benzyl- 1,2,4-oxadiazol-5-yl]- 1*H*-indol-3-yl]ethylamine (L-694,247) and 5-methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1*H*-indole (RU 24969) hemisuccinate were purchased from Tocris (Bioblock Illkirch, France). (−)-4-(dipropylamino)-1,3,4,5-tetahydrobenz-{*c*,*d*}indole-6-carboxamide (LY-228729) was kindly donated by Lilly (Indianapolis, USA); *N*-[2-[4-(2-metho-

Table 2
Affinity of a series of 5-HT_{1A} receptor ligands for the high and low affinity states of the receptor labeled by 0.2 nM [^{3}H]8-OH-DPAT or 0.25 nM [^{3}H] *p*-MPPF + 100 μM Gpp(NH)p, respectively, in membrane preparations from rat hippocampus (results are expressed as pK_i ± S.E.M. of three determinations, and the slope factor is indicated between parentheses)

Compound	[^{3}H]8-OH-DPAT (slope factor)	[^{3}H] *p*-MPPF + Gpp(NH)p (slope factor)	Ratio K_i low/K_i high (95% confidence limits)
5-HT	9.1 ± 0.08 (0.76)	7.0 ± 0.04 (0.79)	110 (36–340)
5-CT	10.0 ± 0.07 (0.83)	8.4 ± 0.07 (0.83)	43 (17–108)
RU 24969	8.9 ± 0.10 (0.82)	7.3 ± 0.13 (0.84)	33 (17–63)
LY-228729	10.3 ± 0.03 (1.16)	8.9 ± 0.01 (0.85)	27 (22–33)
L-694,247	9.1 ± 0.03 (0.88)	7.7 ± 0.03 (0.88)	26 (14–47)
(−)-8-OH-DPAT	9.1 ± 0.09 (0.75)	7.7 ± 0.03 (0.85)	24 (13–44)
(+)-8-OH-DPAT	9.3 ± 0.06 (0.83)	7.9 ± 0.05 (0.79)	23 (15–34)
(±)-8-OH-DPAT	9.1 ± 0.09 (0.76)	7.9 ± 0.06 (0.87)	16 (4.2–58)
WY 50,324	9.5 ± 0.01 (0.95)	8.4 ± 0.03 (1.24)	12 (9.0–16)
Lesopitron	7.5 ± 0.05 (0.82)	6.5 ± 0.02 (0.97)	12 (6.2–22)
Ipsapirone	8.5 ± 0.03 (0.78)	7.5 ± 0.03 (0.95)	9.8 (8.6–11)
Buspirone	8.0 ± 0.01 (0.81)	7.2 ± 0.01 (0.89)	6.9 (6.6–7.2)
(+)Flesinoxan	9.1 ± 0.07 (1.00)	8.3 ± 0.02 (0.91)	6.0 (3.1–12)
Spiroxatrine	9.1 ± 0.03 (0.93)	8.3 ± 0.05 (0.99)	5.8 (4.8–7.0)
(−)Pindolol	8.3 ± 0.06 (0.82)	7.5 ± 0.03 (0.91)	5.8 (4.3–7.9)
NAN-190	9.4 ± 0.01 (0.96)	8.7 ± 0.04 (1.02)	4.3 (2.7–6.7)
BMY 7378	8.8 ± 0.03 (1.00)	8.4 ± 0.02 (1.04)	2.6 (1.6–4.2)
Clozapine	6.6 ± 0.09 (0.90)	6.3 ± 0.06 (0.90)	2.2 (0.82–5.7)
Dihydroergotamine	8.8 ± 0.05 (1.56)	8.9 ± 0.01 (1.34)	0.80 (0.44–1.5)
S-14506	9.4 ± 0.02 (1.56)	9.7 ± 0.03 (1.40)	0.61 (0.39–0.96)
WAY 100635	9.3 ± 0.12 (1.34)	9.9 ± 0.09 (1.30)	0.23 (0.15–0.33)
Spiperone	7.4 ± 0.04 (0.68)	8.3 ± 0.01 (1.27)	0.12 (0.08–0.19)

yphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanearboxamide (WAY 100635) dihydrochloride, S-14506, (+)flesinoxan hydrochloride, lesopitron, ipsapirone hydrochloride, and *N*-(29(4-(2-pyrimidinyl)-1-piperazinyl) ethyl)tricyclo(3.3.1.1(3,7))decane-1-carboxamide (WY 50,324) were synthesized at the Centre de Recherche Pierre Fabre.

3. Results

In rat hippocampal membrane preparations, both [^{3}H]8-OH-DPAT and [^{3}H]*p*-MPPF labeled 5-HT$_{1A}$ receptors with high affinity. The total number of binding sites labeled by [^{3}H]*p*-MPPF was higher than that labeled by [^{3}H]8-OH-DPAT. The addition of 100 μM Gpp(NH)p to the membranes did not change the affinity of [^{3}H]*p*-MPPF or its total number of binding sites (Table 1).

The affinities of a series of 5-HT$_{1A}$ receptor ligands for the high and low affinity states of the receptor labeled by 0.2 nM [^{3}H]8-OH-DPAT and by 0.25 nM [^{3}H]*p*-MPPF in the presence of 100 μM Gpp(NH)p, respectively, are reported in Table 2. Hill coefficient values varied between 0.68 and 1.56 for competition with [^{3}H]8-OH-DPAT binding and between 0.79 and 1.40 for competition with [^{3}H]*p*-MPPF binding in the presence of Gpp(NH)p. In the two experimental conditions, the highest Hill coefficients were obtained with dihydroergotamine and S-14506. Representative examples of the inhibition of [^{3}H]8-OH-DPAT binding and of [^{3}H]*p*-MPPF binding in the presence of 100 μM Gpp(NH)p are shown in Fig. 1. The magnitude of the difference in affinity varied among the compounds tested.

Inhibition of [^{3}H]*p*-MPPF binding in the absence of Gpp(NH)p by the compounds that showed the highest separation of K_i values was fitted better by a two-site model than by a one-site model. Thus, two affinities could be determined for 5-HT ($K_{i1} = 12.6 \pm 4.3$ nM, $K_{i2} = 326 \pm 177$ nM; $n = 3$), 5-CT ($K_{i1} = 0.46 \pm 0.13$ nM, $K_{i2} = 7.2 \pm 3.4$ nM; $n = 3$) and (+)-8-OH-DPAT ($K_{i1} = 2.8 \pm 1.3$ nM, $K_{i2} = 55.7 \pm 30.7$ nM; $n = 3$). For RU 24969, LY-228729 and (−)-8-OH-DPAT, in two out of three experiments, a two-site model fitted the data better than a one-site model ($K_{i1} = 10$ nM, $K_{i2} = 93$ nM; $K_{i1} = 0.13$ nM, $K_{i2} = 791$ nM; and $K_{i1} = 3.5$ nM, $K_{i2} = 39$ nM, respectively), and for (±)-8-OH-DPAT, in one out of three experiments, a two-site model fitted the data better than a one-site model ($K_{i1} = 3.6$ nM, $K_{i2} = 67$ nM).

The highest ''low/high'' affinity ratios were found with 5-HT and 5-CT, which are known to have high intrinsic activity. In contrast, 5-HT$_{1A}$ receptor antagonists, such as WAY 100635 and spiperone, had ratios less than one. Unexpectedly, S-14506 and dihydroergotamine, both agonists with high intrinsic activity also had ratios less than one (0.61 and 0.80, respectively).

Using Spearman's rank order correlation (SigmaStat), the ratios low/high affinity for 5-HT$_{1A}$ receptors were compared with intrinsic activities reported in the literature (Table 3). The correlation of low/high affinity ratios with intrinsic activity depended in part on the study from which the intrinsic activity values were taken. There was a significant, positive rank correlation ($P < 0.001$) between intrinsic activity expressed as E_{max} value from [^{35}S]GTPγS binding in C6-glial cells expressing h5-HT$_{1A}$ receptors (Pauwels et al., 1997) and the ratio of K_i high/low affinity in rat hippocampus. When the low/high affinity ratios were compared with E_{max} values from [^{35}S]GTPγS binding performed in Chinese hamster ovary cells express-

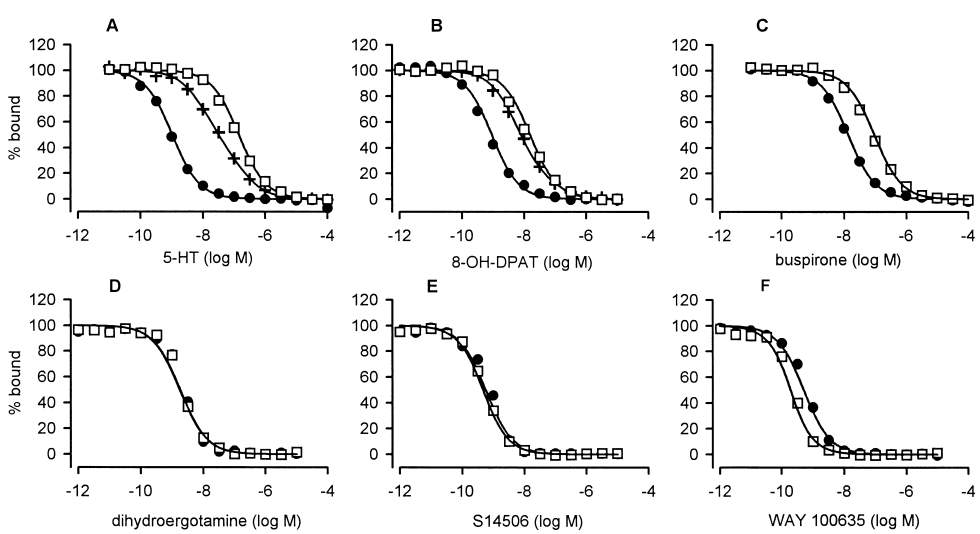

Fig. 1. Inhibition of [^{3}H]8-OH-DPAT and [^{3}H]*p*-MPPF binding in rat hippocampal membranes by various 5-HT$_{1A}$ receptor ligands with different intrinsic activity: (A) 5-HT, (B) (±)-8-OH-DPAT, (C) buspirone, (D) dihydroergotamine, (E) S-14506, and (F) WAY 100635. Each curve is from a representative experiment repeated three times. (●) [^{3}H]8-OH-DPAT, (□) [^{3}H]*p*-MPPF + Gpp(NH)p (both one-site fit), (+) [^{3}H]p-MPPF (two-site fit).

ing human 5-HT_{1A} receptors (CHO-h5-HT_{1A} cells) (Newman-Tancredi et al., 1998), and with E_{max} values from forskolin-stimulated cAMP accumulation in HA7 cells (Koek et al., 1998), the correlation was less strong ($r_s = 0.64$, $P < 0.05$), or not significant ($r_s = 0.39$, NS), respectively. S-14506 was among the compounds examined in the latter two studies, but not in the aforementioned study by Pauwels et al. (1997). When S-14506 was excluded from the analyses, the correlations increased and both were now statistically significant ($P = 0.006$ and 0.013, respectively).

To verify that the binding of S-14506 was: (1) competitive and (2) reversible, saturation experiments were conducted in the presence and the absence of S-14506. Binding parameters of saturation experiments with [^{3}H]8-OH-DPAT alone and in the presence of 1 nM of S-14506 were $K_d = 0.74$ and 1.83 nM; $B_{max} = 14.8$ and 13.9 fmol/mg tissue, respectively ($n = 2$). The K_d was increased and the B_{max} was unchanged, as expected for a competitive and reversible ligand.

The 5-HT_{1A} receptor agonists dihydroergotamine and S-14506 inhibited forskolin-stimulated cAMP accumulation in HA7 cells (IC_{50} values: 5.8 and 2.2 nM, respectively). This inhibition was blocked completely in the presence of pertussis toxin (Fig. 2).

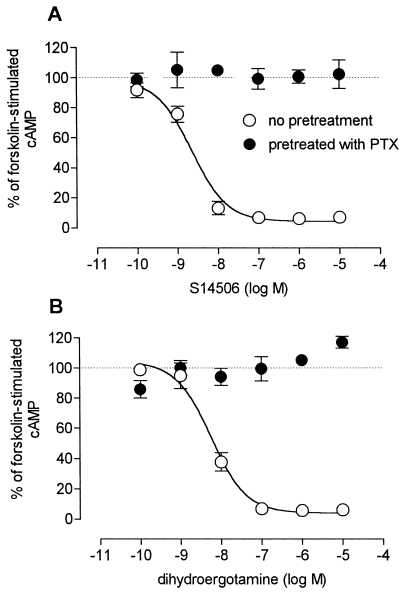

Fig. 2. Inhibition of forskolin-stimulated cAMP accumulation in HA7 cells by: (A) S-14506 and (B) dihydroergotamine in the absence or presence of pertussis toxin. Each point is the mean ± S.E.M. of three experiments, each performed in triplicate.

Table 3
Correlations between K_i low/K_i high ratios and previously reported intrinsic activity values, expressed as % of 5-HT [(A) Pauwels et al., 1997; (B) Newman-Tancredi et al., 1998; (C) Koek et al., 1998] [Spearman's rank order correlations were calculated by means of SigmaStat (SPSS, Chicago)]

Compound	K_i low/ K_i high	E_{max} (A)	E_{max} (B)	E_{max} (C)
5-HT	110	100	100	100
5-CT	43	85	96	
RU 24969	33		95	
LY-228729	27			88
L-694,247	26	98		
(−)-8-OH-DPAT	24	29		
(+)-8-OH-DPAT	23	59		
(±)-8-OH-DPAT	16	41	76	81
WY 50,324	12			79
Lesopitron	12			70
Ipsapirone	9.8	26	49	46
Buspirone	6.9	22	65	49
(+)Flesinoxan	6.0	45	94	93
Spiroxatrine	5.8	27	45	
(−)Pindolol	5.8	22		
NAN-190	4.3			0
BMY 7378	2.6			0
Clozapine	2.2		53	
S-14506	0.61		90	95
WAY 100635	0.23	−2		
Spiperone	0.12	1		
Spearman's rank order correlation		$r_s = 0.88$, $n = 13$, $P < 0.001$	$r_s = 0.64$, $n = 10$, $P = 0.043$	$r_s = 0.39$, $n = 11$, $P = 0.22$
Spearman's rank order correlation (excluding S-14506)			$r_s = 0.80$, $n = 9$, $P = 0.006$	$r_s = 0.73$, $n = 10$, $P = 0.013$

4. Discussion

The present data, which show dihydroergotamine and S-14506 to have high affinity for the G protein-uncoupled state of the 5-HT_{1A} receptor, provide further evidence that S-14506 acts in a manner different from other agonists at 5-HT_{1A} receptors, and extend this finding to dihydroergotamine. Because small low/high affinity ratios were observed not only with antagonists, but also with some agonists (i.e., S-14506 and dihydroergotamine), such ratios are not necessarily predictive of low intrinsic activity. In contrast, large low/high affinity ratios were observed only with strong agonists, suggesting that compounds having large ratios in the present test system are likely to have high intrinsic activity.

The 5-HT_{1A} receptor agonist radioligand [^{3}H]8-OH-DPAT has been used to determine the affinity of agonists and antagonists at 5-HT_{1A} receptors for more than 15 years (Gozlan et al., 1983). In contrast, 5-HT_{1A} receptor antagonist radioligands have not been available until recently, because the existing antagonists were non-selective and, therefore, unsuitable for binding experiments using native membrane preparations. Several selective antagonists have been discovered in the mid-nineties (Fletcher et al., 1993; Forster et al., 1995; Thielen et al., 1996). Among them, [^{3}H] *p*-MPPF is now commercially available (Kung et al., 1996). The binding characteristics of [^{3}H]8-OH-DPAT and

[^{3}H] *p*-MPPF observed here are in agreement with previous published data (Hall et al., 1985; Kung et al., 1996).The B_{max} value for [^{3}H]8-OH-DPAT binding was markedly lower than that obtained with [^{3}H] *p*-MPPF, in support of the existence of two forms of the receptor, with only the high affinity site being labeled by the agonist. Gozlan et al. (1995) reported similar differences in B_{max} values using [^{3}H]WAY 100635 as 5-HT$_{1A}$ receptor antagonist ligand. Neither the K_d nor the B_{max} of [^{3}H] *p*-MPPF were affected by adding Gpp(NH)p to the hippocampal membranes. In contrast, the binding capacity of [^{3}H]8-OH-DPAT has been shown to be decreased by guanine nucleotides (e.g., Hall et al., 1985; Nénonéné et al., 1994; Harikumar and Chattopadhyay, 1998).

Biphasic inhibition by an agonist of binding to receptors labeled with an antagonist radioligand has been used to estimate its intrinsic activity. For example, Leff et al. (1985) showed that dopamine D$_1$ receptor agonists with high intrinsic activity inhibited the binding of an antagonist radioligand in a biphasic manner, and demonstrated that the low/high affinity ratio correlated positively with intrinsic activity in a functional model. Consistent with these results, only 5-HT, 5-CT, and (+)-8-OH-DPAT inhibited [^{3}H] *p*-MPPF binding in a biphasic manner. Thus, using this approach, only 5-HT$_{1A}$ receptor agonists with high intrinsic activity could be identified.

In contrast, by using an agonist to label high affinity sites and an antagonist in the presence of Gpp(NH)p to label the low affinity site of an agonist, it is possible to estimate the intrinsic activity of compounds within a wider range of efficacies. For example, this approach has been used to determine the intrinsic activity of dopamine D$_2$ or 5-HT$_2$ receptor ligands in transfected cells (Lahti et al., 1992; Fitzgerald et al., 1999). For most of the compounds tested here, the separation between their affinity for sites labeled by [^{3}H]8-OH-DPAT and for sites labeled by [^{3}H] *p*-MPPF in the presence of Gpp(NH)p, expressed as a low/high affinity ratio of K_i values (ratios ranging from 110 for the full agonist 5-HT to 0.12 for the antagonist spiperone), is in agreement with their intrinsic activity. Surprisingly, the 5-HT$_{1A}$ receptor agonists, S-14506 and dihydroergotamine, although having high intrinsic activity (see Koek et al., 1998; Newman-Tancredi et al., 1998; Pauwels et al., 1998), had similar high affinities for the G protein-coupled and -uncoupled forms of the receptor. The low/high affinity ratios of the series of compounds tested (except S-14506 and dihydroergotamine) appeared to be predictive of their reported intrinsic activity as measured with [^{35}S]GTPγS binding or with forskolin-stimulated cAMP accumulation in various cell lines expressing h5-HT$_{1A}$ receptors (Pauwels et al., 1997; Koek et al., 1998; Newman-Tancredi et al., 1998). This is evidenced by the significant positive rank correlations between intrinsic activity and low/high affinity ratio obtained with the compounds tested, but excluding S-14506. A similar positive rank correlation was found between intrinsic activity measured by [^{35}S]GTPγS binding in HA7 cells (C. Cosi, unpublished observations) and the low/high affinity ratio for a series of 5-HT$_{1A}$ receptor ligands, and when S-14506 and dihydroergotamine were included in the analysis, the correlation coefficient was decreased.

The unexpectedly high affinity binding to the G protein-uncoupled form of the receptor by S-14506 and dihydroergotamine could result from binding to the receptor in a manner different from those of other agonists. Both S-14506 and dihydroergotamine inhibited binding along very steep curves with slope factors > 1.3 in the two binding conditions, whereas the other high efficacy 5-HT$_{1A}$ receptor agonists had slopes of < 1.3. These high Hill coefficients might reflect positive cooperativity of S-14506 and dihydroergotamine at 5-HT$_{1A}$ receptor binding sites. Stratford et al. (1988) have shown that [^{3}H]dihydroergotamine binding to 5-HT$_{1B}$ receptors was relatively insensitive to G protein inactivation by *N*-ethyl-maleimide. In addition, Sundaram et al. (1995) reported that [^{3}H]lisuride, a structural analogue of dihydroergotamine, could label both the G protein-coupled and -uncoupled forms of 5-HT$_{1A}$ receptors in CHO cells. Thus, it is conceivable that the ergot structure of dihydroergotamine and lisuride is responsible for their unusual binding at different receptors. The possibility that the binding of S-14506 is irreversible or non-competitive could be excluded, because the B_{max} of [^{3}H]8-OH-DPAT was not changed when saturation experiments were conducted in the presence of 1 nM S-14506. A recent study by Lima et al. (1997) demonstrated that there was a strong correlation between the inhibition of [^{3}H]8-OH-DPAT and [^{3}H]S-14506 binding by various 5-HT$_{1A}$ receptor ligands. These authors suggested, however, that the two ligands do not recognize exactly the same sites. Indeed, the B_{max} values of [^{3}H]S-14506 were 65–90% higher than those of [^{3}H]8-OH-DPAT. They also reported that [^{3}H]S-14506 binding was not altered by the addition of GTP or Gpp(NH)p, which agrees with our findings that inhibition of [^{3}H] *p*-MPPF binding by S-14506 was insensitive to Gpp(NH)p.

In terms of functional responses to 5-HT$_{1A}$ receptor activation, there is no evidence that S-14506 and dihydroergotamine activate G proteins different from those activated by other 5-HT$_{1A}$ receptor agonists. Indeed, like other 5-HT$_{1A}$ receptor agonists, they stimulate [^{35}S]GTPγS binding or inhibit forskolin-stimulated cAMP accumulation (Koek et al., 1998; Newman-Tancredi et al., 1998; Pauwels et al., 1998). In addition, the inhibition of forskolin-stimulated cAMP accumulation induced by both compounds was reversed completely by pertussis toxin, as expected for a G$_i$ protein mediated effect.

Taken together, these results suggest that, although the low/high affinity ratio of a ligand for the 5-HT$_{1A}$ receptor generally covaries with its intrinsic activity at this receptor, S-14506 and dihydroergotamine may interact with the 5-HT$_{1A}$ receptor in a manner different from that of other 5-HT$_{1A}$ receptor agonists, likely at the level of the binding

site, rather than because of the involvement of different G proteins.

Acknowledgements

The authors thank Nathalie Danty and Nathalie Leduc for their excellent technical assistance.

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