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Receptor binding characteristics of [^{3}H]NAD-299, a new selective 5-HT$_{1A}$ receptor antagonist

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Abstract

In vitro receptor binding properties of the novel tritiated 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor antagonist (*R*)-3-*N*,*N*-dicyclobutylamino-8-fluoro-[6-^{3}H]-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide ([^{3}H]NAD-299, generic name robalzotan) were evaluated and compared with those of the agonist 8-hydroxy-2-[2,3-^{3}H]di-*n*-(propylamino)tetralin ([^{3}H]8-OH-DPAT). [^{3}H]NAD-299 binding displayed a K_d value of 0.17 nM and a B_{max} value of 26.7 pmol/g wet weight of rat hippocampus. Same binding affinity (K_d = 0.16 nM) was found to cloned human 5-HT$_{1A}$ receptors. Addition of the nonhydrolyzable GTP analog guanylylimidodiphosphate had no effect on the binding characteristics of [^{3}H]NAD-299, while it significantly decreased both the affinity and density of receptors labeled with [^{3}H]8-OH-DPAT. The rank order of potency of various compounds to inhibit [^{3}H]NAD-299 binding is consistent with the labeling of 5-HT$_{1A}$ receptors. This newly developed high-affinity and selective antagonist radioligand provides a valuable tool for studies of 5-HT$_{1A}$ receptors both in vitro and in vivo.

Keywords: 5-HT$_{1A}$ receptor; 5-HT$_{1A}$ receptor antagonist radioligand; [^{3}H]NAD-299; Robalzotan, [^{3}H]8-OH-DPAT (8-hydroxy-2-[2,3-^{3}H]di-*n*-(propylamino)tetralin); Receptor binding, in vitro

1. Introduction

5-Hydroxytryptamine (5-HT) has been implicated in a wide range of physiological functions including control of mood, appetite, cardiovascular function, thermoregulation, motor activity, and sexual and aggressive behavior. Disturbances in serotonergic pathways have also been postulated to play an important role in various neuropsychiatric disorders. Several subtypes of 5-HT receptors have been found and classified according to their pharmacological properties and molecular structure (Humphrey et al., 1993). Among these subtypes the 5-HT$_{1A}$ receptor is one of the targets for antidepressant and anxiolytic drugs (Dourish, 1987; Charney et al., 1990). The 5-HT$_{1A}$ receptors are members of the guanine nucleotide-binding protein (G-protein) coupled receptors with seven transmembrane spanning regions and stimulation of 5-HT$_{1A}$ receptors has been shown to inhibit adenylate cyclase activity.

Until 1995, all radioligands available to study the 5-HT$_{1A}$ receptor were agonists or partial agonists, e.g., 8-hydroxy-2-([2,3-^{3}H]di-*n*-propylamino) tetralin ([^{3}H]8-OH-DPAT). Thus, the labeling was limited mainly to the high-affinity agonist state of the receptor. The propylaminotetralin derivative (*S*)-UH-301 ((*S*)-5-fluoro-8-hydroxy-2-(di-*n*-propylamino)tetralin) of 8-OH-DPAT was the first 5-HT$_{1A}$ receptor antagonist described (Hillver et al., 1990). However, (*S*)-UH-301 had only 8-fold selectivity towards dopamine D$_2$ receptors (Hillver et al., 1990). The phenylpiperazine derivative *N*-*tert*-butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide (WAY-100135) and the more selective (*N*-(2-(1-(4-(2-methoxyphenyl)piperazinyl))-ethyl)-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride) (WAY-100635) were later described as potent and selective 5-HT$_{1A}$ receptor antagonists devoid of intrinsic activity (Cliffe et al., 1993; Fletcher et al., 1993; Fletcher et al., 1994; Khawaja et al., 1995). These compounds, in particular WAY-100635, became important tools to further characterize the 5-HT$_{1A}$ receptor function.

Recently, a structurally different antagonist NAD-299 ((*R*)-3-*N*,*N*-dicyclobutylamino-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrogen (2*R*,3*R*)-tartrate, generic name robalzotan) was introduced and shown to possess a high 5-HT$_{1A}$ affinity and even higher selectivity

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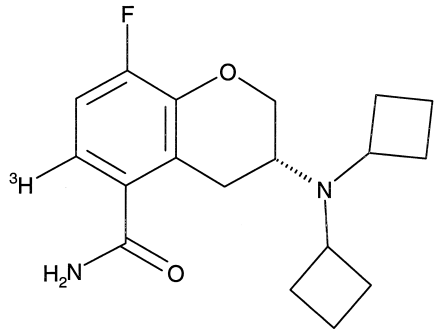

Fig. 1. The chemical structure of [^{3}H]NAD-299 ([^{3}H]robalzotan).

than WAY-100635 (Johansson et al., 1997). NAD-299 behaves like an antagonist both in vitro in 5-HT receptor mediated inhibition of cyclic AMP accumulation in GH4ZD10 cells expressing rat 5-HT_{1A} receptors, and in vivo by antagonizing various agonist-induced biochemical and behavioral responses. Thus, it antagonized 8-OH-DPAT-induced responses on 5-HT turnover, hypothermia, corticosterone secretion and inhibition of passive avoidance behavior (Johansson et al., 1997).

In the present study, the free base of NAD-299 was tritiated (Fig. 1) as a novel 5-HT_{1A} receptor antagonist radioligand. The binding characteristics of this newly developed radioligand were investigated and compared with those of the 5-HT_{1A} agonist [^{3}H]8-OH-DPAT. Rat hippocampal membranes and Chinese Hamster Ovary (CHO) cells expressing human cloned 5-HT_{1A} receptors were used for in vitro receptor binding assays.

2. Materials and methods

2.1. Compounds

(*R*)-3-*N*,*N*-Dicyclobutylamino-8-fluoro-[6-^{3}H]-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide ([^{3}H]NAD-299), with specific activity 22 Ci/mmol was synthesized at Astra Arcus, Södertälje, Sweden. 8-Hydroxy-2-(*N*,*N*-[2,3-^{3}H]dipropylamino)[1,2,3-^{3}H]-1,2,3,4-tetrahydronaphthalene ([^{3}H]-8-OH-DPAT), with specific activity 129.5, 120 and 154.3 Ci/mmol were purchased from Du Pont-NEN, Boston, MA, USA. Flesinoxan was obtained from Duphar, Weesp, Holland. Buspirone, serotonin (5-HT) and guanylylimidodiphosphate (Gpp(NH)p) were purchased from Sigma, St. Louis, MO, USA. Ipsapirone was obtained from Troponwerke, Cologne, Germany. (±)8-OH-DPAT and (±)-pindolol were from Research Biochemicals, Natick, MA, USA. WAY-100635 (*N*-(2-(1-(4-(2-methoxyphenyl)-iperazinyl))ethyl)-*N*-(2-pyridinyl)cyclohexanecarboxamide rihydrochloride) was obtained from Wyeth Research, Maidenhead, UK. NAD-299 ((*R*)-3-*N*,*N*-Dicyclobutylamino - 8- fluoro- 3,4- dihydro- 2*H*-1-benzopyran - 5-carboxamide hydrogen (2*R*,3*R*)-tartrate monohydrate) and NDL-249 ((*R*)-3-(*N*-cyclopentyl-*N*-propylamino)-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrochloride) were synthesized at Astra Arcus. Other chemicals were obtained from commercial sources and were of analytical grade.

2.2. Tissue preparation

Male Sprague–Dawley rats weighing 150–220 g (obtained from B&K Universal, Sollentuna, Sweden) and housed at standard laboratory conditions (18–22°C, 40–80% humidity, 15–20 air changes/h, artificial 12/12 dark/light cycle, lights on at 0600 h) with free access to food (Brood Stock Feed R36 Lactamin; Stockholm, Sweden) and tap water, were used. The animals were acclimatized to laboratory conditions for at least 5 days and housed in groups of five animals per transparent macrolon cage with bedding material consisting of aspen wood cuttings (B&K Universal, Nittendal, Norway). The rats were decapitated and the hippocampi were dissected out on ice, frozen and stored at −70°C until the day of experiment. The tissue was thawed and homogenized in 50 mM Tris–HCl containing 10 mM EDTA (pH 7.4) using an Ultra-Turrax followed by centrifugation for 10 min at 48 000 × *g* and 5°C. The pellet was resuspended in 50 mM Tris–HCl and recentrifuged. The final pellet was homogenized and suspended in assay buffer containing 50 mM Tris–HCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$, pH 7.4 (unless otherwise stated). In order to remove endogenous serotonin the hippocampal membranes were preincubated for 10 min at 37°C, whereafter pargyline was added to give a final concentration of 0.25 μM.

2.3. Cells

CHO cells, expressing human 5-HT_{1A} receptors were obtained from Dr. Philip G. Strange (Department of Biosciences, The University of Kent at Canterbury, Kent, UK). The CHO cells were grown and the cell membranes prepared as described by Sundram et al. (1993).

Protein concentration was measured by the method of Markwell et al. (1978).

2.4. Radioligand binding assays

The kinetic binding studies of [^{3}H]NAD-299 were performed in triplicates at 30°C using rat hippocampal membranes (1.5 mg w.w.) and 0.3 nM radioligand diluted in ascorbic acid (final concentration 0.01%). The final assay volume was 2 ml. In the association studies, total and nonspecific binding were determined at different time points (0.5–150 min). Nonspecific binding was determined as that bound in the presence of 10 μM WAY-100635. The dissociation was initiated after an association for 30

min by the addition of 10 μM WAY-100635 and the binding was measured during 0–140 min.

Saturation studies with rat hippocampal membranes (1.5 mg w.w.) were carried out in duplicate at 30°C for 2 h with the concentration range 0.01 to 5 nM of [^{3}H]NAD-299 and 0.04 to 16 nM of [^{3}H]8-OH-DPAT. The final assay volume was 2 ml (unless otherwise stated).

Saturation studies with cloned 5-HT$_{1A}$ receptors were carried out in duplicate at 30°C for 70 min with a final volume of 2 ml and 0.01 mg protein for the [^{3}H]NAD-299 binding and with a final volume of 0.5 ml and 0.024 mg protein for the [^{3}H]8-OH-DPAT binding. The assay buffer contained 50 mM Tris–HCl and 4 mM $MgCl_2$, pH 7.4 at room temperature.

In competition experiments, 0.3 nM [^{3}H]NAD-299 was incubated with 10–12 concentrations (2 points/log unit) of the competing drug diluted in ascorbic acid (final concentration 0.01%). The reactions were started by addition of membranes (1.5 mg w.w.) and stopped by rapid filtration through Whatman GF/B glass fiber filters pretreated with 0.3% polyethylenimine and subsequent washing with cold buffer (5 mM Tris–HCl, pH 7.4) using a Brandel cell harvester. Scintillation cocktail was added and the radioactivity determined in a Packard 2500TR liquid scintillation counter at about 50% efficiency.

Competition experiments with [^{3}H]8-OH-DPAT were carried out in duplicate at 37°C for 45 min with a final volume of 0.5 ml. The radioligand (1 nM) and the compounds (10 concentrations with 2 points/log unit) were diluted in ascorbic acid to give a final concentration of 0.01%. Nonspecific binding was determined as that bound in the presence of 100 μM 5-HT. The reactions were started by the addition of rat hippocampal membranes (2.5 mg w.w.) in 50 mM Tris–HCl buffer (pH 7.4) containing 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ and stopped by rapid filtration through Whatman GF/B glass fiber filters and subsequent washing with cold Tris–HCl buffer (50 mM) using Brandel harvester.

2.5. Calculations

Data from the saturation and competition experiments were analyzed using the iterative non-linear curve-fitting program LIGAND (Munson and Rodbard, 1980). One- and two-site curve fitting was tested in all experiments. The K_d values used in the calculation of the K_i values were determined under the corresponding assay conditions. The Hill coefficients (n_H) were calculated for each individual experiment.

Statistical comparisons were carried out by using Student's unpaired *t*-test or for multiple tests one-way analysis of variance (ANOVA) followed by a post-hoc Dunnett's *t*-test.

The slope, k_{obs}, of the pseudo first order association kinetics and the dissociation rate constant k_{-1} were determined as described by Weiland and Molinoff (1981). The actual association rate constant (k_{+1}) was then determined by conversion of k_{obs} using the equation:

$$k_{+1} = (k_{obs} - k_{-1})/L \quad (L = \text{radioligand concentration})$$

The half-time ($T_{1/2}$) of association and dissociation was calculated as $\ln 2/k_{obs}$ and $\ln 2/k_{-1}$, respectively.

The estimated time for equilibration was calculated by multiplying the half-time of dissociation by 5 (Hulme and Birdsall, 1992).

3. Results

3.1. Association and dissociation kinetics of [^{3}H]NAD-299 binding

The time-course of the binding of [^{3}H]NAD-299 to rat hippocampal 5-HT$_{1A}$ receptors is shown in Fig. 2. The forward rate constant (k_{+1}) for the [^{3}H]NAD-299 binding at 30°C was $0.49 \pm 0.28 \times 10^9$ M^{-1} min^{-1} ($n = 3$) with a half-time association value ($T_{1/2}$) of 3.77 ± 0.10 min. The

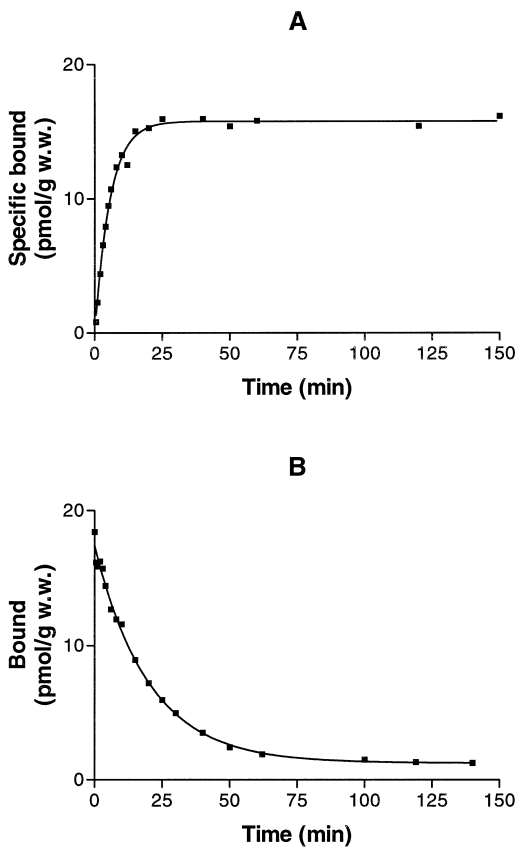

Fig. 2. Association (A) and dissociation (B) kinetics of [^{3}H]NAD-299 binding to rat hippocampal membranes. (A) Membranes corresponding to 1.5 mg original wet tissue were incubated with 0.3 nM [^{3}H]NAD-299 at 30°C for various times. The nonspecific binding was determined with 10 μM WAY-100635. Each value is the mean of triplicate determinations. (B) Membranes were incubated with [^{3}H]NAD-299 for 30 min, whereafter 10 μM WAY-100635 was added and the incubation was continued for various times. Each value is the mean of triplicate determinations.

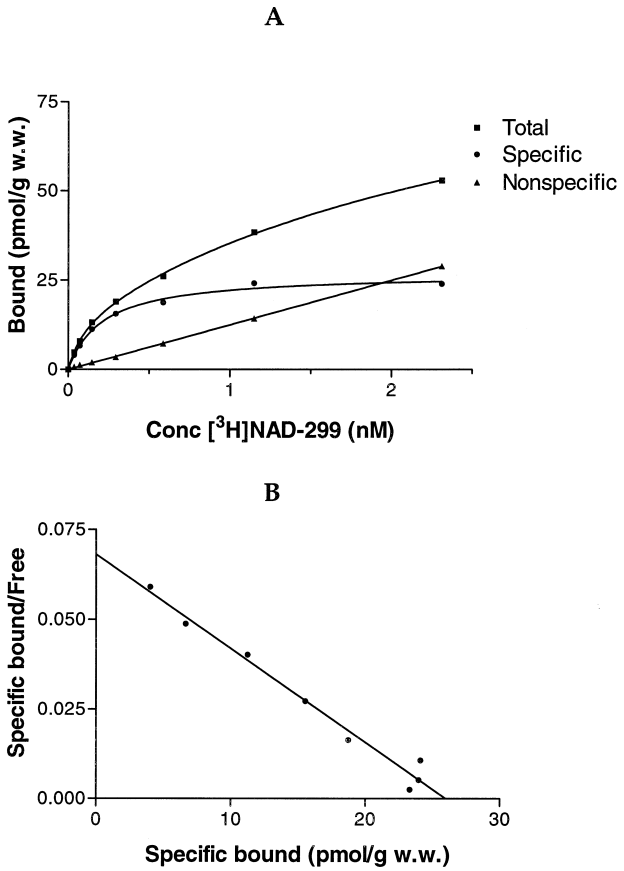

Fig. 3. [^{3}H]NAD-299 binding to rat hippocampal 5-HT$_{1A}$ receptors. The radioligand binding studies were performed as described under Section 2. The nonspecific binding was determined as that bound in the presence of 10 μM WAY-100635. Each value is the mean of duplicate determinations. (A) Representative saturation binding curve for [^3H]NAD-299 ($K_d = 0.20$ nM, $B_{max} = 26.0$ pmol/g w.w.). (B) The corresponding Scatchard plot ($K_d = 0.19$ nM, $B_{max} = 26.0$ pmol/g w.w.).

dissociation rate constant (k_{-1}), determined by addition of 10 μM WAY-100635, was 0.056 ± 0.004 min^{-1} ($n = 3$). The dissociation was complete after 90 min with a half-time dissociation value ($T_{1/2}$) of 12.4 ± 0.8 min. Thus the estimated time needed to achieve binding equilibrium was 62 min (5×12.4 min) (Hulme and Birdsall, 1992). The specific binding was stable for at least 160 min indicating that neither the receptors nor the radioligand degraded during the incubation time. The calculated ratio of the rate constants (k_{-1}/k_{+1}) gave an estimated K_d value of 0.12 $\pm$ 0.01 nM ($n = 3$). The data support the first-order association and dissociation kinetics.

3.2. Equilibrium binding studies

Fig. 3A shows a representative saturation binding curve of [^{3}H]NAD-299 to rat hippocampal 5-HT$_{1A}$ receptors and Fig. 3B the corresponding Scatchard plot. [^{3}H]NAD-299 bound with high affinity and saturability with the K_d and B_{max} values of 0.17 ± 0.01 nM and 26.7 ± 1.6 pmol/g w.w., respectively ($n = 8$). Thus, the K_d value from equilibrium binding studies agrees with the kinetically derived K_d value. Scatchard analysis of specific [^{3}H]NAD-299 binding resulted in a linear plot consistent with a non-cooperative single class of binding site. Under similar experimental conditions [^{3}H]8-OH-DPAT bound with the K_d value of 0.54 ± 0.05 nM and B_{max} value of 21.5 ± 1.2 pmol/g w.w. ($n = 7$) which is significantly lower (80%) than the number of binding sites labelled with [^{3}H]NAD-299 ($P < 0.05$; Student's t-test).

The receptor binding characteristics of [^{3}H]NAD-299 to cloned human 5-HT$_{1A}$ receptor expressed in CHO cells were also investigated. The K_d value obtained was 0.16 ± 0.01 nM and the B_{max} value was 2150 ± 190 fmol/mg protein ($n = 3$). Thus, the K_d value of [^{3}H]NAD-299 binding to cloned human 5-HT$_{1A}$ receptor is in good agreement with that obtained in rat hippocampal tissue. [^{3}H]8-OH-DPAT displayed under similar experimental conditions a K_d value of 0.54 ± 0.12 nM and a B_{max} value of 1400 ± 180 fmol/mg protein ($n = 3$). Thus, the B_{max} value of [^{3}H]8-OH-DPAT binding to cloned human

Table 1
Effects of Na^+ and Gpp(NH)p on [^{3}H]NAD-299 and [^{3}H]8-OH-DPAT binding to the rat hippocampal 5-HT$_{1A}$ receptor

Buffer	[^{3}H]NAD-299		[^{3}H]8-OH-DPAT	
	K_d (nM)	B_{max} (pmol/g w.w.)	K_d (nM)	B_{max} (pmol/g w.w.)
A	0.17 ± 0.01^a	27.8 ± 2.1	0.46 ± 0.07	22.9 ± 0.5
A + G	0.21 ± 0.03	28.9 ± 2.6	0.76 ± 0.15	15.0 ± 1.2^b
A + NMDG	0.47 ± 0.05^b	25.0 ± 2.7	1.38 ± 0.31	18.9 ± 1.7
A + NMDG + G	0.72 ± 0.12^b	25.7 ± 1.3	$3.13 \pm 0.22^{a,b}$	$11.0 \pm 1.5^{a,b}$
A + Na^+	0.48 ± 0.08^b	27.9 ± 3.4	1.29 ± 0.22	17.2 ± 0.5^b
A + Na^+ + G	0.75 ± 0.10^b	30.0 ± 3.1	$5.02 \pm 0.72^{a,b,c}$	$10.3 \pm 0.1^{a,b,c}$

The radioligand binding studies were carried out and the equilibrium dissociation constants (K_d) and receptor densities (B_{max}) were calculated as described in Section 2. The results are means $\pm$ S.E.M. of three experiments (unless otherwise stated) performed in parallel. Buffer A contained: 50 mM Tris–HCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$, pH 7.4. The additions were: G: 0.1 mM Gpp(NH)p; NMDG: 120 mM; Na^+: 120 mM NaCl. Statistical comparisons were performed using one-way ANOVA followed by Dunnett's post-test with A + NMDG ($^aP < 0.05$) or A ($^bP < 0.05$) as control. $^c n = 2$ (one experiment with no detectable specific binding is excluded).

5-HT_{1A} receptor was significantly lower (65%) than the B_{max} value of [^{3}H]NAD-299 binding ($P < 0.05$; Student's *t*-test).

3.3. Effects of ions and guanine nucleotides

Table 1 summarizes the effects of the nonhydrolyzable guanine nucleotide analog Gpp(NH)p and NaCl on [^{3}H]NAD-299 and [^{3}H]8-OH-DPAT binding. In these studies, 120 mM *N*-methyl-D-glucamine (NMDG) was added instead of NaCl in order to maintain the ionic strength. As can be seen, the K_d values of both [^{3}H]NAD-199 and [^{3}H]8-OH-DPAT were elevated 2- to 3-fold by the addition of 120 mM NMDG although the increase of the K_d value of [^{3}H]8-OH-DPAT was not statistically significant. Notably, NaCl did not have any additional effect as compared to the effect of NMDG on either the K_d or B_{max} values of the two radioligands.

As expected, the addition of Gpp(NH)p did not have any effect on the density of receptors labelled by [^{3}H]NAD-299 while the amount of receptors labelled by [^{3}H]8-OH-DPAT was significantly reduced (see Table 1). In the presence of NaCl and Gpp(NH)p only 34% of the receptors labelled by [^{3}H]NAD-299 was labelled with [^{3}H]8-OH-DPAT as compared to 76% in the absence of NaCl and Gpp(NH)p.

Addition of 0.1 mM Gpp(NH)p slightly elevated the K_d value of [^{3}H]8-OH-DPAT, while it did not have any statistically significant effect on the affinity of [^{3}H]NAD-199 (Table 1).

3.4. Competition studies

Table 2 summarizes the K_i values of a number of agonists, partial agonists and antagonists at rat hippocampal 5-HT_{1A} receptors labelled with [^{3}H]NAD-299 and [^{3}H]8-OH-DPAT. The Hill coefficients of all the competition curves were close to 1 and the competition curves were best fitted to an one-site model. The K_i values of various compounds obtained with [^{3}H]NAD-299 were in good agreement ($r = 0.97$) with those obtained with [^{3}H]8-OH-DPAT binding suggesting that the radioligands label the same receptor (Table 2). This is further confirmed by the cross-competition studies shown in Fig. 4. As can be seen, the cold ligands were able to compete with [^{3}H]NAD-299 to the level of 10 μM WAY-100635 (defined as 0%) and with [^{3}H]8-OH-DPAT to the level of 100 μM 5-HT (defined as 0%). The Hill coefficients for both ligands were close to 1. Moreover, the K_i values for unlabelled NAD-299 and 8-OH-DPAT were in good agreement with the K_d values of the radioligands presented in Table 1.

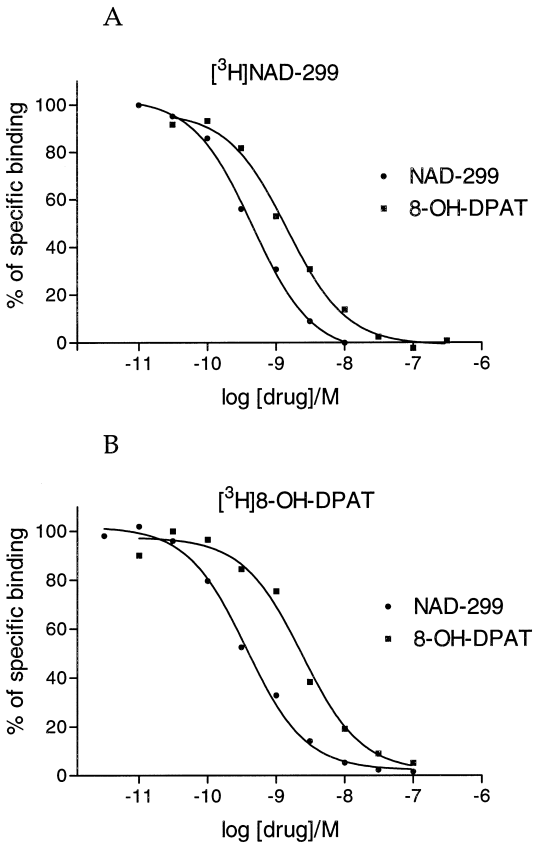

Fig. 4. Representative inhibition curves of NAD-299 and 8-OH-DPAT for [^{3}H]NAD-299 (A) and [^{3}H]8-OH-DPAT (B) binding to rat hippocampal 5-HT_{1A} receptors. Competition experiments were performed as described in Section 2 and the curves are the best fits to one-site model.

Table 2
Potencies of various compounds to inhibit [^{3}H]NAD-299 and [^{3}H]8-OH-DPAT binding to rat hippocampal 5-HT_{1A} receptors

Compound	[^{3}H]NAD-299		[^{3}H]8-OH-DPAT	
	K_i (nM)	n_H	K_i (nM)	n_H
Serotonin	0.81 ± 0.09	0.80 ± 0.01	1.48 ± 0.35	0.90 ± 0.03
Ipsapirone	2.48 ± 0.18[a]	1.07 ± 0.12	2.77 ± 0.81[a]	0.93 ± 0.01
(±)8-OH-DPAT	0.69 ± 0.10[a]	0.87 ± 0.08	0.96 ± 0.10	0.78 ± 0.12
Flesinoxan	0.57 ± 0.01	1.09 ± 0.11	0.33 ± 0.04	1.19 ± 0.14
Buspirone	6.82 ± 0.95[a]	0.93 ± 0.03	4.75 ± 0.29	0.98 ± 0.01
(±)-Pindolol	9.13 ± 0.42	0.97 ± 0.07	9.30 ± 0.60[a]	0.78 ± 0.06
NDL-249	1.19 ± 0.26	1.05 ± 0.09	1.50 ± 0.05	0.87 ± 0.07
NAD-299	0.18 ± 0.03	0.98 ± 0.07	0.23 ± 0.01	0.98 ± 0.06
WAY-100635	0.18 ± 0.05	0.94 ± 0.04	0.12 ± 0.04	1.07 ± 0.04

The competition studies were performed and the K_i values were calculated as described in Section 2 using a buffer containing 50 mM Tris–HCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$, pH 7.4. The results are means ± S.E.M. from three to four independent experiments if not otherwise stated ([a] $n = 2$).

4. Discussion

The pharmacological properties of 5-HT_{1A} receptors have been extensively investigated using the selective agonist [^{3}H]8-OH-DPAT as a radioligand (Hall et al., 1985;

Hoyer et al., 1985), since no antagonist radioligand has been available until 1995 when [^{3}H]WAY-100635 was introduced (Gozlan et al., 1995; Khawaja et al., 1995). Biphasic binding curves with [^{3}H]8-OH-DPAT have been reported by several authors (Mongeau et al., 1992; Chamberlain et al., 1993; Nénonéné et al., 1994), supporting the existence of both high- and low-affinity agonist states of the 5-HT$_{1A}$ receptor. The studies were carried out over a broad range of [^{3}H]8-OH-DPAT concentrations from 0.5 nM to more than 50 nM with K_{high} ranging between 0.5 and 0.8 nM and K_{low} between 8 and 36 nM. However, under the buffer conditions used and within the concentration range of radioligand used (0.04 to 16 nM) in this study, we did not observe any indications of two binding sites with [^{3}H]8-OH-DPAT. Thus, the binding site labeled by [^{3}H]8-OH-DPAT corresponds to the high-affinity agonist state of the receptor.

It is well known that guanine nucleotides decrease the proportion of receptors in the high-affinity state, whereas divalent cations, especially Mg^{2+}, promote the formation of the high-affinity state (Birnbaumer and Birnbaumer, 1995). Furthermore, Na^+ has been shown to be an important regulator of several G_i coupled receptors such as α_2-adrenoceptor (Nunnari et al., 1987) and dopamine D$_2$ receptors (Neve, 1991). It decreases the affinity of agonists for the receptor as well as increases the ability of agonists to inhibit adenylyl cyclase. In our studies, Na^+ did not have any effect on the B_{max} or K_d values of [^{3}H]8-OH-DPAT binding. Thus, Na^+ does not seem to be as strong regulator of the 5-HT$_{1A}$ receptor as it is of other G_i coupled receptors. In addition, we found that in the presence of both Na^+ and Gpp(NH)p, a fraction (38%) of the receptors was still detected by [^{3}H]8-OH-DPAT. This may be due to the presence of the divalent cations (Mg^{2+}, Mn^{2+} and Ca^{2+}) in the binding assay. However, preliminary studies (results not shown) and previous results from several other groups have shown that a small portion of 5-HT$_{1A}$ receptors is still detected after GTP addition, even in the absence of divalent cations (Hall et al., 1985; Emerit et al., 1990, 1991; Mongeau et al., 1992; Nénonéné et al., 1994; Khawaja et al., 1995). Thus, the effects of guanine nucleotides on the 5-HT$_{1A}$ receptors are different from those on dopamine D$_2$ receptors (Malmberg and Mohell, 1995) and β-adrenoceptor receptors (Kent et al., 1980), where no high-affinity agonist binding was detected in the presence of 0.1 mM Gpp(NH)p. The molecular mechanism underlying these differences remains to be investigated.

NAD-299 has been shown to be a selective and potent 5-HT$_{1A}$ receptor antagonist (Johansson et al., 1997). Among the receptors tested with in vitro receptor binding (5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_3$, 5-HT$_6$, 5-HT$_7$, 5-HT uptake site, α_1-, α_2- and β-adrenoceptor, muscarinic and nicotinic acetylcholine, dopamine D$_1$, D$_{2A}$, and D$_3$, GABA$_A$ and GABA$_B$, NMDA, AMPA, benzodiazepine, histamine H$_1$ and H$_2$, galanin, CCK$_A$, CCK$_B$, neuropeptide Y, somatostatin and glycine receptors and the strychnine-insensitive glycine site on the NMDA receptor) NAD-299 bound in nM range only to the 5-HT$_{1A}$ receptor (K_i = 0.6 nM) and with more than 400 times lower affinity to the α_1- and β-adrenoceptors (K_i = 260 and 340 nM, respectively) (Johansson et al., 1997).

In the present study, the receptor binding characteristics of the tritiated form of NAD-299 were investigated. We found that the B_{max} values in rat hippocampus of the antagonist [^{3}H]NAD-299 obtained are in agreement with those shown in the studies using [^{3}H]WAY-100635 (Gozlan et al., 1995; Khawaja et al., 1995) and [^{125}I]p-MPPI (Kung et al., 1995) as radioligands. As expected, ions and guanine nucleotides did not have any effect on [^{3}H]NAD-299 binding, which is consistent with its properties as an antagonist.

In conclusion, the results of in vitro receptor binding studies of [^{3}H]NAD-299 in rat hippocampus show that it labels both G-protein coupled and uncoupled 5-HT$_{1A}$ receptors with similar affinity, which is consistent with the characteristics of an antagonist radioligand. The highly selective 5-HT$_{1A}$ receptor antagonist [^{3}H]NAD-299 provides a valuable tool for studies of 5-HT$_{1A}$ receptors both in vitro and in vivo.

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