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European Journal of Pharmacology 497 (2004) 147–154

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[^3H] A-369508 ([2-[4-(2-cyanophenyl)-1-piperazinyl]-*N*-(3-methylphenyl) acetamide): an agonist radioligand selective for the dopamine D_4 receptor

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Received 15 June 2004; accepted 24 June 2004

Abstract

Tritiation of the dopamine D_4 receptor selective agonist A-369508 ([2-[4-(2-cyanophenyl)-1-piperazinyl]-*N*-(3-methylphenyl) acetamide) has provided a radioligand for the characterization of dopamine D_4 receptors. [^3H] A-369508 binds with high affinity to the major human dopamine D_4 receptor variants $D_{4.2}$, $D_{4.4}$ and $D_{4.7}$ (K_d=1.7, 4, and 1.2 nM, respectively). It also binds to the rat dopamine D_4 receptor, (K_d=4.4 nM), implying similar binding affinity across human and rat receptors. A-369508 shows >400-fold selectivity over D_{2L}, >350-fold selectivity over 5-HT_{1A} and >700–1000-fold selectivity over all other receptors tested. Agonist activity determined by inhibition of forskolin-induced cAMP in Chinese hamster ovary cells transfected with the human dopamine $D_{4.4}$ receptor (EC_{50}=7.5 nM, intrinsic activity=0.71) indicates that A-369508 is a potent agonist at the human dopamine D_4 receptor. Similar data was observed in other functional assays. [^3H] A-369508 binds to a single, high affinity site on membranes containing the human dopamine $D_{4.4}$ receptor. When compared to the D_2-like antagonist [^3H] spiperone, competition binding for agonists like dopamine and apomorphine were 2–10-fold more potent with [^3H] A-369508, while the antagonists clozapine, haloperidol and L-745870 bind with similar affinity to both ligands. Binding to rat brain regions demonstrated that the most abundant area was cerebral cortex (51.2 fmol/mg protein) followed by hypothalamus, hippocampus, striatum and cerebellum. [^3H] A-369508 is a useful tool to define the localization and physiological role of dopamine D_4 receptors in central nervous system and can facilitate measuring accurate affinities (K_i) for structure/activity relationship studies designed to identify dopamine D_4 receptor selective agonists.

Keywords: Dopamine; D_4 receptor; Spiperone; A-369508

1. Introduction

Dopamine, the predominant catechol neurotransmitter in the brain, exerts its actions via two classes of G-protein coupled receptors: the Gs-coupled D_1-like family (D_1 and D_5) and the Gi/o-coupled D_2-like family (D_2, D_3, D_4) (Missale et al., 1998). The dopamine D_4 receptor is expressed predominantly within the central nervous system (Oak et al., 2000) and despite low abundance relative to the D_2 receptor, localization in cortex suggests an important functional role (Ariano et al., 1997; Khan et al., 1998). The high affinity of clozapine for this receptor and the efficacy of clozapine as an atypical antipsychotic led to the proposal of the dopamine D_4 receptor played a key role in the pathophysiology of schizophrenia (Tarazi and Baldessarini, 1999). However, the lack of efficacy of dopamine D_4 receptor selective antagonists in human clinical trials has compelled a reexamination of this hypothesis (Hrib, 2000).

Ligand affinity for the dopamine D_4 receptor has utilized the antagonist spiperone (Asghari et al., 1995). While

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doi:10.1016/j.ejphar.2004.06.049

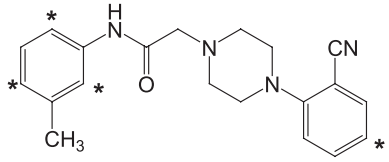

Fig. 1. Structure of A-369508. Asterisks denote the sites of tritium in the radiolabeled compound.

spiperone potently binds to the receptor with reliable, stable binding in membranes derived from cloned receptor expressing cells, the lack of selectivity of spiperone for a single D_2-like dopamine receptor renders this ligand unsuitable for whole brain studies. Furthermore, no studies have addressed whether agonist radioligands distinguish in competition assays between agonists and antagonists at the dopamine D_4 receptor. Using the D_2 receptor, different affinities up to 500-fold can be detected for antagonists versus agonists in competition binding using [^3H] spiperone versus [^{125}I]-PIPAT {(*R*,*S*)-2′ -*trans*-7-hydroxy-2-[*N*-*n*-propyl-*N*-(3′ -iodo-2′ -propenyl)-amino]tetralin} or [^3H] U-86170 [(*R*)-(-)-5-(dipropylamino-5,6-dihydro-1*H*,4*H*-imidazo[5,1-ij]quinolin-2-(1*H*)-one] (Chang et al., 2002, Lahti et al., 1992).

Unlike dopamine D_4 receptor selective antagonists, the reports of dopamine D_4 receptor agonists in the literature are limited (Glase et al., 1997). In this report, we have synthesized and characterized A-369508 (Fig. 1), a potent dopamine D_4 receptor full agonist ligand, demonstrated its selectivity for the dopamine D_4 receptor, compared binding with spiperone against cloned receptors and examined [^3H] A-369508 binding in rat brain regions.

2. Materials and methods

2.1. Radioligand binding assay

Human dopamine $D_{4.4}$ receptor-transfected human embryonic kidney (HEK)-293 cells ($hD_{4.4}$-G_{qo5} HEK-293) were prepared as reported (Moreland et al., 2004). The rat dopamine D_4 receptor was cloned as described and transfected into HEK-293 cells (Gazi et al., 2000). Both cell lines were cultured in Dulbecco's minimum essential media (DMEM) supplemented with 10% fetal calf serum, 1 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Rockville, MD). For membrane preparation, the cells were seeded into a Cell Factory (VWR, Plainfield, NJ) and the confluent cells were rinsed with PBS and detached with cell dissociation buffer (Invitrogen). The resulting cell suspension was centrifuged and the pellet homogenized using a Polytron for 10s in 50 mM Tris–HCl, pH 7.4. Membrane aliquots were frozen in liquid nitrogen and stored at −80 °C until use. Membranes containing recombinant human dopamine $D_{4.2}$ and $D_{4.7}$ receptors were obtained from Perkin Elmer Life Sciences, Boston, MA.

Binding assays were initiated by addition 250 μl of membrane to 200 μl of [^3H] A-369508 (88.1 Ci/mmol) and were incubated at room temperature for 1 h. Nonspecific binding was determined in the presence of 10 μM PD 168077 (RBI-Sigma) or 10 μM haloperidol. There was no difference in binding assays with cloned receptors with PD 168077 or haloperidol. In brain binding assays (see below), haloperidol was used. The incubation buffer consisted of 50 mM Tris–HCl, pH 7.4, 5 mM KCl, 120 mM NaCl, 5 mM $MgCl_2$, and 1mM EDTA. In competition binding studies, stock solutions of agonists or antagonists were prepared with 0.1% ascorbic acid and 0.5% 3-isobutyl-5-methylxanthine (IBMX) in the buffer and diluted to final concentrations with binding buffer. The final concentration for [^3H] A-369508 was 2 nM. The reaction was terminated by rapid filtration through UniFilter-96 GF/B filers presoaked in 0.5% polyethyleneimine, using a Filtermate Harvester (Packard, Meriden, CT). Filters were washed three times with 1 ml of ice cold 50 mM Tris–HCl, pH 7.4. Radioactivity was measured using a TopCount Microplate Scintillation Counter (Packard). Proteins were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. Assays using [^3H] spiperone (Amersham) were carried out in similar fashion except using 10 μM haloperidol to determine noncompetitive binding and incubating for 2 h at room temperature with a final radioligand concentration of 0.2 nM.

Radioligand competitive binding assays for human dopamine D_{2L} and D_3 receptors were carried out using membranes derived from HEK-293 cells expressing recombinant receptors (gift of Dr. Liliane Unger, Abbott Ludwighaven) using [^3H] spiperone (Amersham) (Moreland et al., in press). Radioligand competitive binding assays for rat serotonin 5-HT_{1A} (5-HT_{1A}) receptor were carried out using membranes derived from rat cortex expressing recombinant receptors and the agonist [^3H] 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) (NE Nuclear, Boston, MA) (De Vry et al., 1998).

Competition curves for nonradioactive compounds were analyzed by nonlinear regression curve-fitting program (Prism, GraphPad Software, San Diego, CA). All assays were performed in triplicate and IC_{50} values were converted to K_i values by the method of Cheng and Prusoff (1973).

2.2. D_4 binding in rat brain regions

Rat cerebellum, cortex, hippocampus, hypothalamus, striatum and spinal cord were prepared from freshly sacrificed male rats and snap frozen in liquid nitrogen (Pel-freeze, Rogers, AR). Membranes were prepared and binding conditions (buffer and time of incubation) were the same as described above for $D_{4.4}$ transfected cells. Protein concentrations varied from 0.1 to 0.3 mg/tube and 10 μM haloperidol was used to assess specific binding.

2.3. cAMP determinations

Chinese hamster ovary (CHO) cells expressing the human dopamine $D_{4.4}$ receptor (obtained from Dr. Hubert Van Tol, University of Toronto) were grown as reported (Asghari et al., 1995). cAMP was measured using the adenylate cyclase activation FlashPlate Assay (Perkin Elmer Life Sciences). CHO cells were plated in 75 cm^2 flasks 1–2 days before the experiment and grown to 90% confluency. The cells were washed with D-PBS w/o Ca^{++}/Mg^{++} and harvested using a nonenzymatic cell dissociation buffer (Sigma-Aldrich). The cells were washed in 35 ml DMEM, and the cell pellet suspended at 1×10^6 cell/ml in stimulation buffer containing 100 μM IBMX. The cell suspension (50 μl/well) was incubated at room temperature for 20 min with 50 μl of compound (0.0001–10 μM, dissolved in D-PBS, 0.004% ascorbate) in the presence of 10 μM forskolin (Invitrogen). Detection buffer was added and the plate read on a Packard TopCount after a 2-h incubation at room temperature. cAMP data were expressed as a percentage of forskolin-stimulated levels. EC_{50}s were calculated by analyzing the data by nonlinear regression curve-fitting program (Prism, GraphPad Software).

2.4. Agonist-stimulated Europium-GTP (GTP-Eu) binding assay

GTP-Eu binding assay was carried out as previously reported (Hsieh et al., 2004) (DELFIA GTP-binding kit, Perkin Elmer Life Science). In the preliminary experiments, the assay conditions were optimized, and nonspecific binding was measured in the presence of 100 μM GTP-γS. Different concentrations of ligand (0.1–10,000 nM) were incubated with 8 μg of membranes containing human dopamine $D_{4.2}$ receptors (Perkin Elmer Life Science) in GTP-Eu binding buffer containing 50 mM HEPES, pH 7.4, 10 mM MgCl, 25 mM NaCl, Saponin 100 μg/ml, 1 μM GDP and 10 nM GTP-Eu at room temperature for 40 min. The reaction was terminated by rapid filtration and the filter was washed three times with 225 μl of ice-cold washing solution in a vacuum manifold. The plate was measured using time-resolved fluorometer, 1420 VICTOR™ Multi-label Counter. Basal GTP-Eu was measured in the absence of ligand. Percentage of stimulation was calculated as 100 times the difference between the counts of agonist and 10 μM of dopamine–stimulated binding. EC_{50} values were calculated by analyzing the data by nonlinear regression curve-fitting program (Prism, GraphPad Software).

2.5. Materials

DPBS, neomycin (G418), hygromycin B and tissue culture reagents were from Invitrogen/Life Technologies (Rockville, MD). All other chemicals were from Sigma unless otherwise noted. PD168077 {*N*-[4-(2-cyanophenyl)-piperazin-1-ylmethyl]-3-methyl-benzamide} (Glase et al., 1997) and CP226269 {5-fluoro-2-(4-pyridin-2-yl-piperazin-1-ylmethyl)-1*H*-indole} (Zorn et al., 1997) were synthesized by Abbott Laboratories. [^3H] Spiperone (100 Ci/mmol) was obtained from Amersham. A-369508 {2-[4-(2-cyanophenyl)-1-piperazinyl]-*N*-(3-methyl phenyl) acetamide} was prepared at Abbott Laboratories. The tetratritiated derivative of A-369508 was prepared from [2-[4-(4-bromo-2-cyanophenyl) piperazin-1-yl]-*N*-(2,4,6-tribromo-3-methylphenyl) acetamide] by hydrogenolysis of the aryl bromides with tritium gas (Matulenko et al., in press). The specific activity of tetratritiated A-369508 was 88.1 Ci/mmol.

3. Results

3.1. Receptor selectivity

A-369508 is highly selective for dopamine D_4 receptors, binding with an affinity of 4.04±0.69 nM to the cloned human dopamine $D_{4.4}$ receptor (Tables 1 and 2). The selectivity of A-369508 was determined for more than 70 different neurotransmitter receptors and ion channels (Cerep, Paris, France). Those sites showing specific binding <1 μM as well as binding to dopamine receptors are shown in Table 1. Binding of A-369508 to human $D_{4.4}$ is greater than 400-fold selective over other dopamine receptors (D_1, >2475; D_{2L}, 434; D_3 1022; D_5, >2475) and greater than

Table 1
Receptor selectivity of A-369508 and PD168077

Receptor	Ligand	A-369508		PD168077	
		K_i (nM)	Selectivity	K_i (nM)	Selectivity
Human $D_{4.4}$	[^3H] A-369508	4.0	1	11.9	1
Human D_1	[^3H] SCH23390	>10000	>2475	4600	387
Human D_{2L}	[^3H] Spiperone	1753	434	1280	108
Human D_3	[^3H] Spiperone	4127	1022	2300	193
Human D_5	[^3H] SCH23390	>10000	>2475	4500	378
Human α_1	[^3H] Prazosin	3000	743	100	8
Rat 5-HT_{1A}	[^3H] 8-OH-DPAT	1219	302	600	50
Human 5-HT_{1B}	[^{125}I] Cyanopindolol	>10000	>2475	300	25

Data were determined either by CEREP (average of triplicate samples) or as described in Materials and methods for human D_{2L}, D_3, $D_{4.4}$ or rat 5-HT_{1A} (rat cortex). Selectivity D_4=K_i (receptor in question)/K_i $D_{4.4}$.

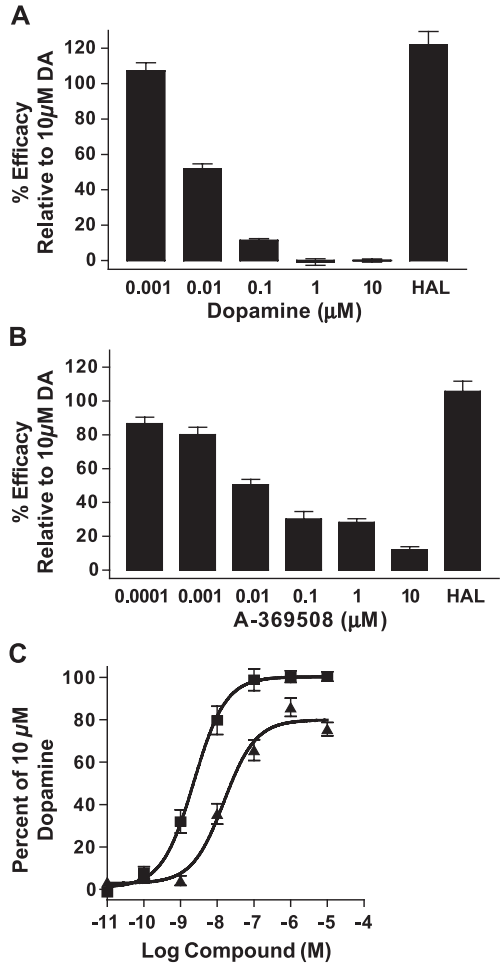

Fig. 2. Agonist activity of A-369508. Inhibition of forskolin-induced cAMP by dopamine and A-369508 activation of human dopamine $D_{4.4}$ receptors. (A) Dopamine inhibition of forskolin-induced cAMP. HAL- 1 μM dopamine plus 10 μM haloperidol. (B) A-369508 inhibition of forskolin-induced cAMP. HAL- 1 μM A-369508 plus 10 μM haloperidol. GTP-γ-S-Eu shifts with A-369508. (C) Agonist–stimulation of GTP-γ-S binding activity by dopamine (■) and A-369508 (▲) in recombinant human $D_{4.2}$ dopamine receptor. Data for each concentration point were normalized to the maximal effect of dopamine (10 μM) and presented as the mean (±S.E.M.) of the triplicate assays (n=3).

300-fold selective over 5-HT_{1A} receptors (K_i=1219±274 nM). Despite weak affinity for the human α_1-adrenoceptor (K_i=3000 nM), A-369508 does not bind to any of the more than 70 different neurotransmitter receptors and ion channels examined (K_i<10000 nM). PD168077 has been reported as a selective D_4 receptor agonist (Glase et al., 1997); however, in this study we also find substantial affinity to α-adrenoceptor and serotonin 5-HT_{1A} and 5-HT_{1B} receptors (100, 600 and 300 nM, respectively; Table 1). A-369508 is an improvement over PD168077 with no affinity for these receptors K_i>1000 nM. Therefore, A-369508 represents a highly selective D_4 receptor ligand and is a good candidate for a radiolabeled reagent.

3.2. Agonist activity of A-369508

The agonist activity of A-369508 was determined by measuring the inhibition of forskolin-induced cAMP synthesis in CHO cells transfected with the human dopamine $D_{4.4}$ receptor. In this assay, dopamine potently inhibited forskolin-induced cAMP (EC_{50}=10.7±1.1 nM, n=8; Fig. 2A) consistent with published reports (Asghari et al., 1995). A-369508 also potently inhibited forskolin-induced cAMP (EC_{50}=6.6±1.3 nM, n=4; Fig. 2B) and this inhibition could be blocked with 10 μM haloperidol, consistent with agonist activity through dopamine receptors. Compared to 10 μM dopamine, A-369508 has an intrinsic activity of 0.71 this assay. Agonist activity was also determined using GTP-γ-S binding. When assayed using the cloned human $D_{4.2}$ receptor, dopamine dose dependently increased GTP-γ-S-Eu binding (EC_{50}=3.4±1.6 nM, n=5, Fig. 2C). A-369508 in this assay also had potent agonist activity (EC_{50}=15.3±1.3 nM, with an intrinsic activity of 0.8; n=5). Utilizing a calcium influx assay in human $D_{4.4}$, $G\alpha_{qo5}$ stably cotransfected cells (Moreland et al., in press), A-369508 also exhibited potent agonist activity (EC_{50}=7.7±0.6 nM, with an intrinsic activity of 0.8; n=10). Dopamine in this assay had EC_{50} = 2.2±0.2 nM (n=10). In three different assays of agonist activity, A-369508 is a potent agonist with an intrinsic activity 0.71–0.8 as compared to dopamine (1.0).

3.3. Optimization of assay, time course of binding

The time course of binding of [^3H] A-369508 or [^3H] spiperone to membranes from HEK293 cells expressing the human dopamine $D_{4.4}$ receptor was examined as described in Materials and methods. The saturable specific binding of [^3H] A-369508 reached a maximum at 1 h at 25 °C and was stable for up to 5 h (Fig. 3). In addition, analysis of samples of [^3H] A-369508 did not reveal any decomposition of the compound in the presence of membranes up to 5 h. For

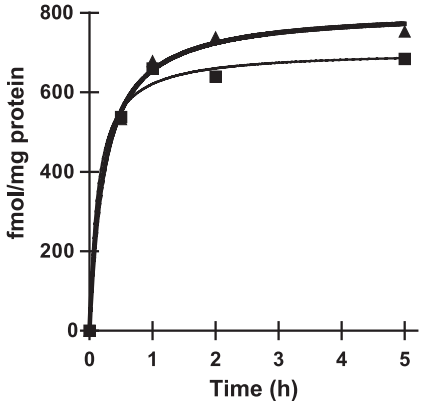

Fig. 3. Time course of binding of [^3H] A-369508 and [^3H] spiperone. Radiolabeled A-369508 (4.0 nM, ■) or spiperone (0.1 nM, ▲) was incubated with membranes from cells expressing human dopamine $D_{4.4}$ receptor and binding determined as described in the Materials and methods. Data represents the mean±standard deviation for n=3 determinations.

subsequent assays, 1 h incubation was used. There was no specific binding of [^3H] A-369508 to HEK-293 cells lacking D_4 receptors. Use of PD168077 or haloperidol to reveal nonspecific binding showed no difference in membranes with cloned human D_4 receptors. Consistent with reports in the literature, [^3H] spiperone binding was optimal by 2 h in this assay (Fig. 2) (Asghari et al., 1995). Spiperone binding was also stable up to 5 h. Binding assays with [^3H] spiperone as the competing ligand were carried out for 2 h at 25 °C.

3.4. A-369508 binding to human dopamine D_4 receptor variants

The human dopamine D_4 receptor is expressed by 20 different alleles with most of these variants differing in the number of 48 bp repeats within the third intracellular loop (Oak et al., 2000). Approximately 96% of the human populations express the $D_{4.2}$, $D_{4.4}$ or $D_{4.7}$ receptor and $D_{4.4}$ is the most common variant (where 2, 4 and 7 are the number of repeats). We examined binding of [^3H] A-369508 to membranes prepared from cells expressing the three most abundant human dopamine D_4 receptor variants. A representative experiment is shown in Fig. 4. In this assay using membranes containing cloned human dopamine $D_{4.4}$ receptor, specific binding accounts for 80% of the signal. As shown in Table 2, [^3H] A-369508 binds with high affinity to the human dopamine $D_{4.2}$, $D_{4.4}$ and $D_{4.7}$ receptors (K_d=1.7, 4, and 1.2 nM, respectively). [^3H] A-369508 also binds to rat dopamine D_4, receptor (K_d=4.4nM), implying similar binding affinity across human and rat receptors. The B_{max} for all four receptor preparations contained comparable receptor numbers/cell (~3000 fmol/mg/protein, Table 2).

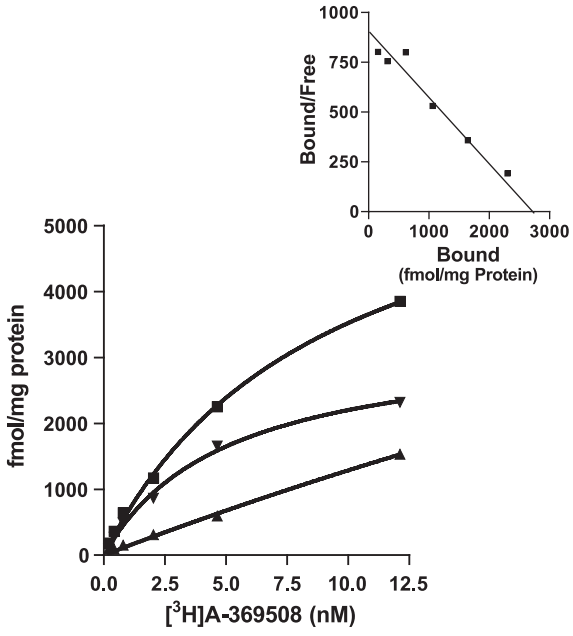

Fig. 4. [^3H] A-369508 binding to rat cortical membranes. Membranes from rat cortex were incubated with increasing concentrations of [^3H] A-369508 and binding determined as described in the methods. Data represent mean for experiments performed in triplicate. Specific binding was determined in the presence of haloperidol. ■: total binding, ●: specific binding and ▲: nonspecific binding. K_d in this experiment was 3.7 nM and B_{max}=47.5 fmol/mg protein.

Table 2
K_d for A-369508 on different cloned D_4 receptors

Receptor	K_d (nM)	B_{max} (fmol/mg protein)
Human $D_{4.2}$	1.68±0.28	3282±435
Human $D_{4.4}$	4.04±0.69	2773±303
Human $D_{4.7}$	1.22±0.19	2688±223
Rat D_4	4.35±0.43	3063±176

Saturation binding of [^3H] A369508 was carried out on membrane preparations derived from cells expressing the appropriate receptor as described in Materials and methods. Data represents the mean±S.E.M. for n=4 determinations.

3.5. Comparison of [^3H] A-369508 and [^3H] spiperone competition binding on human D_4 receptor variants

Using [^3H] A-369508 as the ligand, competitive binding of known dopaminergic agonists and antagonists was determined using membranes prepared from cells expressing human dopamine $D_{4.2}$, $D_{4.4}$ and $D_{4.7}$ receptors. Displacement of [^3H] A-369508 binding by dopamine, apomorphine and the dopamine D_4 receptor selective agonist ligands PD168077 and CP226269 is shown in Table 3. Dopamine competed with high affinity for all three variants ranging from 9.8 to 56.2 nM. There was no difference in binding affinity between the human dopamine $D_{4.2}$ and $D_{4.7}$ receptors while binding to the human dopamine $D_{4.4}$ receptor was five times less potent. Apomorphine shows a similar trend but to a lesser degree. CP226269 and PD168077 showed little difference in competition across the human dopamine D_4 receptor variants, having potencies of 12.8 nM or less. The results for D_2-like receptor antagonist haloperidol and dopamine D_4 receptor selective antagonist L-745870 (3-[4-(4-chlorophenyl)-piperazin-1-ylmethyl]-1H-pyrrolo[2,3-b]pyridine) showed little difference in competition binding across the human dopamine D_4 receptor variants. Clozapine bound most potently to the $D_{4.4}$ receptor with twofold less affinity for $D_{4.2}$ and $D_{4.7}$ receptors.

[^3H] spiperone bound with high affinity to all three human dopamine D_4 receptor variants with K_d values consistent with previous reports (0.01, 0.03 and 0.02 nM for $D_{4.2}$, $D_{4.4}$ and $D_{4.7}$, respectively) (Asghari et al., 1995). The results of competition binding experiments for the same agonists and antagonists examined with A-369508 are shown in Table 3 (data in parentheses). These binding data agree well with reports in the literature for apomorphine, PD168077, CP226269, haloperidol, clozapine and L-745870. Dopamine bound to all three variants with potencies ranging from 112 to 134 nM and no difference among the three receptors. The same trend was observed for

Table 3
Competition binding with [^3H] A-369508 for agonists and antagonists with human $D_{4.2}$, $D_{4.4}$ and $D_{4.7}$ receptors

Compound	Human $D_{4.2}$ K_i (nM)±S.E.M.	Human $D_{4.4}$ K_I (nM)±S.E.M.	Human $D_{4.7}$ K_I (nM)±S.E.M.
Dopamine	11.6±0.6 (112.0±8.4)	56.2±9.1 (134.0±20.0)	9.8±1.3 (120.0±4.0)
Apomorphine	1.1±0.1 (8.1±0.4)	4.0±0.5 (8.9±0.4)	1.2±0.2 (9.3±0.7)
PD168077	10.9±2.7 (9.2±1.4)	11.9±1.1 (22.3±0.1)	12.8±0.9 (30.0±1.0)
CP226269	1.5±0.1 (2.4±0.7)	0.8±0.1 (3.2±0.2)	2.0±0.3 (5.6±0.9)
Haloperidol	3.0±0.3 (1.6±0.04)	1.9±0.3 (1.4±0.04)	4.7±0.5 (2.7±0.1)
Clozapine	71.7±8.1 (41.6±0.4)	27.9±1.8 (28.4±0.4)	62.1±3.9 (50.2±1.0)
L-745870	0.48±0.02 (0.67±0.07)	0.37±0.01 (0.44±0.05)	0.40±0.01 (0.53±0.02)

Data represent the mean±S.E.M. for n=4 determinations. Comparison is shown with competition for the same compounds with [^3H] spiperone (in parentheses).

apomorphine and CP226269. PD168077 did show twofold differences in binding to human dopamine $D_{4.2}$ over $D_{4.4}$ and $D_{4.7}$ receptors when competed with spiperone. The results for D_2-like antagonist haloperidol and D_4-selective antagonist L-745870 also showed little difference in competition binding across the human dopamine D_4 variants. Clozapine bound most potently to the $D_{4.4}$ receptor with twofold less affinity for $D_{4.2}$ and $D_{4.7}$ receptors.

In comparing the results with agonist radioligand [^3H] A-369508 and [^3H] spiperone, the general trend is that agonists bind more potently with [^3H] A-369508 than [^3H] spiperone to the respective human dopamine D_4 receptor variants. However, antagonists show little difference between the two radioligands (Table 3). With the four agonists, this is most pronounced with the dopamine and apomorphine. The exception to this is PD168077 binding to the human dopamine $D_{4.2}$ receptor with no difference in affinity between [^3H] spiperone and [^3H] A-369508. For the antagonists, haloperidol, clozapine and L-745870 show less than twofold differences between the two radioligands. Clozapine bound most potently with both radioligands to the human dopamine $D_{4.4}$ receptor preparation but showed few differences when comparing at human dopamine $D_{4.4}$ and $D_{4.7}$ receptors.

3.6. Characterization of [^3H] A-369508 binding to rat brain membranes

Initial experiments carried out using PD168077 to block nonspecific binding led to an overestimation of the total number of sites. Upon further examination, it was found that this binding was due to a heat-stable component that is probably a brain lipid or carbohydrate (data not shown). Several compounds were tested to block nonspecific sites including L-745870 and haloperidol. Both compounds yielded similar results and haloperidol was used, leading to saturable, specific binding revealing a B_{max}=51.2±3.1 fmol/mg rat cortical protein and a K_d=3.7±0.8 nM (Table 4, Fig. 5) despite that only 10% of binding was specific. The high nonspecific binding may reflect a low density of receptors as well as ligand binding to nonprotein components in the rat cortex. Binding of [^3H] A-369508 to selected brain regions is shown in Table 4. The most abundant D_4 binding with this ligand is cortex, hippocampus and hypothalamus followed by striatum and cerebellum. No specific binding could be detected in the

Table 4
[^3H] A-369508 binding to rat brain regions

Rat brain region	[^3H] A-369508 binding (fmol/mg protein±S.E.M.)	K_d (nM)
Cortex	51.2±3.1	3.7±0.8
Hippocampus	52.4±8.5	4.3±1.8
Hypothalamus	49.0±3.8	3.7±0.3
Striatum	15.3±5.3	0.8±0.1
Cerebellum	6.1±1.7	1.3±0.3
Spinal Cord	No specific binding	–

Data represents the mean±S.E.M. for n=3 determinations.

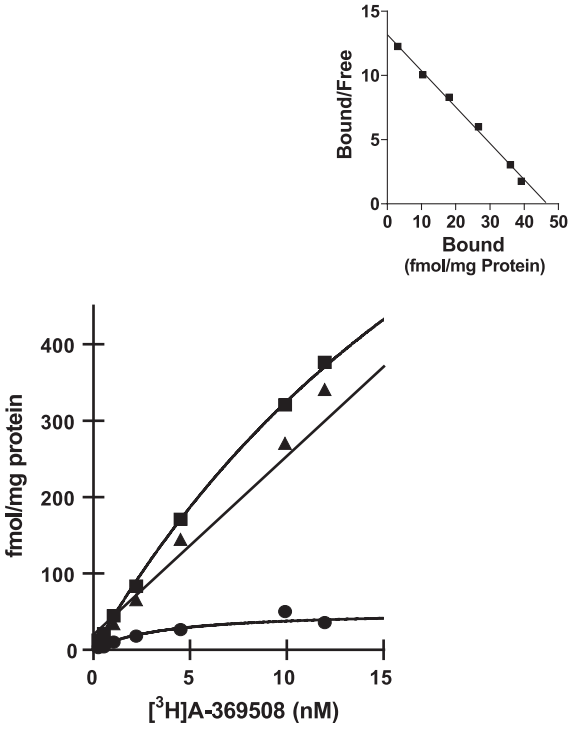

Fig. 5. [^3H] A-369508 binding to cloned human dopamine $D_{4.4}$ receptor. Membranes from cells expressing human dopamine $D_{4.4}$ receptor were incubated with increasing concentrations of [^3H] A-369508 and binding determined as described in Materials and methods. Data represent mean for experiments performed in triplicate. ■: Total binding, ●: specific binding and ▲, ▼: nonspecific binding. K_d in this experiment was 3.3 nM and B_{max} 2600 fmol/mg protein.

spinal cord. The K_d values for [^3H] A-369508 binding in all rat brain regions examined are comparable to binding to the cloned rat dopamine D_4 receptor (Table 4).

4. Discussion

In this report, we describe the pharmacological characterization of the dopamine D_4 receptor selective agonist radioligand [^3H] A-369508. A-369508 bound selectively with D_4 and showed weak affinity for other D_2-like receptors (>1500 nM) as well as more than 70 other neurotransmitter receptors and ion channels (>10000 nM). This is an improvement over the D_4-selective agonist PD168077 that in this study also exhibited adrenoceptor and serotonergic binding. A-369508 has an intrinsic activity of 0.71–0.88 as a dopamine D_4 receptor agonist as compared to dopamine in three different assays of dopamine D_4 activity. [^3H] A-369508 exhibited a high affinity for the three major variants of the human dopamine D_4 receptor as well as rat dopamine D_4 receptors (1.2–4.4 nM) and bound at a single high affinity site. This ligand also displaced dopaminergic agonists as well as antagonists in binding assays and was used to demonstrate D_4 receptors in rat brain regions. The high degree of receptor selectivity of this compound as well as its agonist quality may provide a unique tool for the characterization of D_4 agonists as well as dopamine receptors in the brain.

In comparing competition binding with [^3H] A-369508 and [^3H] spiperone, the general trend was that agonists such as dopamine and apomorphine bound more potently competing with [^3H] A-369508 than [^3H] spiperone while antagonists such as haloperidol and L-746870 showed little difference in affinity in competition assays with the two ligands. While these differences with the human dopamine D_4 receptor are less than observed with the human dopamine D_2 receptor (Lahti et al., 1992), this distinction may be due to agonist binding to G-protein activated receptor. This phenomenon was first demonstrated with the β-adrenoceptor (Williams and Lefkowitz, 1977) and forms the foundation of the two state model of G-protein receptor activation (Kenakin, 2002). Characterization of agonists at this receptor may be facilitated by use a selective agonist radioligand, allowing more accurate determination of affinities of compounds for the dopamine D_4 receptor as has been recognized in structure/activity relationship studies for the human dopamine D_{2L} receptor (van Vliet et al., 1996).

Specific binding of [^3H] A-369508 in rat cortex revealed a low density of high affinity D_4 receptors (K_d=3.7 nM, B_{max}=51.2 fmol/mg protein). This K_d is comparable to binding to membranes prepared from HEK293 cells stably transfected with rat dopamine D_4 receptor (K_d=4.4 nM). Specific binding was also demonstrated with approximately B_{max} ~50 fmol/mg protein in hypothalamus and hippocampus consistent with qualitative results from immunohistochemical reports (Ariano et al., 1997; Khan et al., 1998). Some investigators have used nonselective D_2-like radioligands such as nemonapride in the presence of dopamine D_2/D_3 receptor antagonist raclopride to demonstrate dopamine D_4 receptor binding indirectly (Defagot et al., 2000; Tarazi and Baldessarini, 2000). Utilizing dopamine D_4 receptor gene knock-out mice and raclopride subtraction of nemonapride binding, a high nonspecific binding component was observed but D_4 receptors were estimated at 25 fmol/mg in the mouse cortex (Defagot et al., 2000). Other investigators using the same techniques in rats found 10–15 fmol/mg protein binding depending on the cortical region and the age of the animal (Tarazi and Baldessarini, 2000). It is clear that a direct determination of dopamine D_4 receptors is desirable as subtractive measures are only as good as the non-selective radioligand and antagonist used (Kula et al., 1999).

Dopamine is known to play an important role in sexual function (Moreland et al., 2001). Apomorphine, a potent dopamine D_2-like receptor agonist, potentiates penile erection in rodents, probably through the action of dopamine D_4 receptors (Hsieh et al., 2004). It has been recently demonstrated that selective D_4 agonists such as ABT-724 (2-[(4-pyridin-2-ylpiperazin-1-yl) methyl]-1*H*-benzimidazole) are important in mediating penile erection and may provide a novel treatment for erectile dysfunction (Brioni et al., 2004). In this report, the presence of dopamine D_4 receptors in hypothalamus is particularly notable as the paraventricular nucleus of the hypothalmus is important in sexual function and the central control of penile erection (Hsieh et al., 2004; Moreland et al., 2001). This suggests that dopamine D_4 receptor selective agonists could be used to treat erectile dysfunction. A role in cognition and attention deficit disorder has also been proposed (Hrib, 2000). Thus, the development of dopamine D_4 receptor selective agonists may provide unique therapeutic opportunities as well as research tools to understand dopamine D_4 receptor function in the brain.

In conclusion, radiolabeled A-369508 may provide a useful tool to define the localization and physiological role of dopamine D_4 receptors in central nervous system. Use of this radioligand should provide a more relevant K_d values and facilitate measuring accurate affinities (K_i) for structure/activity relationship studies designed to identify dopamine D_4 receptor selective agonists.

Acknowledgements

The authors thank Dr. Marlon Cowart for preparation of CP226269 and Mrs. Karen St. George and Dr. John Darbyshire for the analysis of [^3H] A-369508 samples to determine stability in the binding assay.

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