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Identification and Pharmacological Characterization of [^{125}I]L-750,667, a Novel Radioligand for the Dopamine D_4 Receptor

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SUMMARY

We identified a novel azaindole derivative, L-750,667, that has high affinity (K_i = 0.51 nM) and >2000-fold selectivity for D_4 dopamine receptors compared with its activity at D_2 and D_3 dopamine receptors. L-750,667 had little affinity for rat D_1/D_5 dopamine receptors, σ binding sites, or 5-hydroxytryptamine$_{1A}$ or 5-hydroxytryptamine$_2$ receptors. In functional studies, L-750,667 exhibited high affinity antagonist activity at D_4 receptors, reversing dopamine (1 μM)-induced inhibition of cAMP accumulation in human embryonic kidney (HEK) cells expressing the human D_4 receptor (hD4 HEK) with an EC_{50} value of 80 nM. The radioiodinated form of L-750,667 bound specifically to the human dopamine D_4 receptor expressed in HEK cells and saturation analysis revealed a single high affinity binding site for [^{125}I]L-750,667 (K_d = 0.16 ± 0.06 nM). The maximum number of binding sites (B_{max}) estimated using [^{125}I]L-750,667 in hD4 HEK cells was 251 ± 71 fmol/mg, which correlated well with the *B*max value determined using [^{3}H]spiperone (227 ± 83 fmol/mg) in the same membrane preparations. The pharmacological profile of [^{125}I]L-750,667 binding to hD4 HEK cells was evaluated using known dopamine receptor agonists and antagonists. The rank order of potencies for dopamine receptor agonists was dopamine > quinpirole > 6,7-aminodihydroxytetralin > 5,6-aminodihydroxytetralin. Dopamine receptor antagonists also showed high affinity, with a rank order of haloperidol > chlorpromazine > domperidone > (+)-butaclamol > (−)-sulpiride = (+)-sulpiride > (+)-SCH23390 > (−)-butaclamol. [^{125}I]L-750,667 bound to D_4 receptors in a stereoselective manner with (+)-butaclamol showing higher activity than its respective enantiomer (−)-butaclamol. These results show that [^{125}I]L-750,667 is a novel, highly selective radioligand for dopamine D_4 receptors and may be used to investigate the dopamine D_4 receptor population in the central nervous system.

Dopamine receptors have been implicated in the etiology of a number of diseases of the central nervous system, including schizophrenia and Parkinson's disease. In 1979, Kebabian and Calne (1) first suggested the existence of multiple dopamine receptors (defined as D_1 and D_2), each with a specific pharmacology and regional distribution. Drugs used in the successful treatment of schizophrenia from a variety of chemical classes have a common property of possessing high affinity antagonist activity at D_2 receptors. There is a good correlation between the plasma concentration of clinically effective doses of neuroleptic agents and their D_2 receptor affinity (2). However, neuroleptic agents also produce a number of mechanism-related side effects, including hyperprolactinemia and extrapyramidal side effects, which are presumably mediated by D_2 receptor antagonism in the pituitary and caudate, respectively (3).

The advent of molecular biological techniques led to the cloning of members of the dopamine receptor family, including the D_1 (4) and the D_2 (5) receptors. In recent years, it has become apparent that at least three novel dopamine receptors exist, two of which show close homology to the D_2 receptor: the D_3 receptor [identified by Sokoloff *et al.* (6)] and the D_4 receptor [first described by Van Tol *et al.* (7)]. The D_5 receptor shows close sequence homology and pharmacological similarity to the D_1 receptor (8).

The D_4 receptor is of particular interest because of its high affinity for the atypical neuroleptic clozapine (7). Clozapine is effective in schizophrenic patients who are refractory to other neuroleptics, and it displays a reduced propensity for extrapyramidal side effects. Clozapine has activity at a range of neurotransmitter receptors (9), but it has proved to be difficult to determine the mechanism of its improved effectiveness. One hypothesis is that the unique clinical profile of clozapine is related to its high affinity for D_4 receptors. Seeman (2) reported that in contrast to other neuroleptics, the plasma levels achieved after clinically effective doses of clozapine seem to show a higher correlation with their D_4 than with their D_2 affinity. The idea that D_4 receptors may be involved in schizophrenia was first suggested on the basis of studies in postmortem brain tissue in which D_4 receptors

ABBREVIATIONS: DMEM, Dulbecco's modified Eagle's medium; PEI, polyethylenimine; IBMX, isobutylmethylxanthine; CHO, Chinese hamster ovary; HEK, human embryonic kidney; ADIN, aminodihydroxytetralin.

were reported to be elevated 6-fold in the human caudate of schizophrenics compared with control subjects (10). This observation was potentially very interesting, but there is some controversy concerning this finding. In particular, in the absence of a D_4-selective radiolabel, the D_4 receptor density has been measured indirectly using a subtraction technique with [^{3}H]emonapride (which binds to D_2, D_3, and D_4 receptors) and [^{3}H]raclopride (with concentrations limited for binding to D_2 and D_3 receptors only). A number of investigators have tried to confirm this finding, again using indirect methodologies, with the result that some reports claim to confirm an elevation of D_4 receptors in postmortem brain of schizophrenics (11, 12), whereas others report no difference between brain tissue from schizophrenics and age-matched controls (13). Clearly, a D_4-selective radioligand is required to directly measure dopamine D_4 receptors in the brain.

In the current study, we describe the profile of L-750,667 [3-(4-(4-iodophenyl)piperazine-1-yl)methyl pyrrolo[2,3-*b*]pyridine; Fig. 1], a highly selective D_4 receptor antagonist that was found to be suitable as a radiolabel for D_4 receptors.

Experimental Procedures

Materials. DMEM was obtained from Gibco Life Technologies (Eragny, France). L-(+)-Ascorbic acid and haloperidol (Haldol) were obtained from Janssen Pharmaceuticals (Antwerp, Belgium). PEI, apomorphine, IBMX, and trichloroacetic acid were purchased from Sigma Chemical (Poole, Dorset, UK).

Synthesis of [^{125}I]L-750,667. The detailed synthesis of L-750,667 has been reported previously (14). [^{125}I]L-750,667 (2000 Ci/mmol) was prepared through custom synthesis at Amersham International (Buckinghamshire, UK) using an iodine exchange reaction.

Cell culture. The expression of the human D_2 receptor in CHO cells and the human D_3 and D_4 receptors in HEK cells has been previously described (6, 7). The human D_2 (short) cell line was obtained from Dr. Mike Graziano (Merck Sharp and Dohme Research Laboratories, Rahway, NJ). The human D_3 cDNA was obtained from Dr. P. Sokoloff (INSERM), and the human D_4 gene/cDNA hybrid construct was obtained from Dr. O. Civelli (Dept. of Cell Biology and Anatomy, Vollum Institute, Oregon Health Sciences University, Portland, OR). Stable hD3 and hD4 HEK cell lines were made by Dr. G. McAllister (Merck Sharp and Dohme Research Laboratories, Harlow, UK). Cells were grown at 37° in a humidified atmosphere of 95% air/5% CO_2 in DMEM containing 10% fetal bovine serum supplemented with 1% glutamine.

[^{3}H]Spiperone binding assay. Clonal cell lines stably expressing the human D_2, D_3, or D_4 receptor were harvested in phosphate-buffered saline and then lysed through homogenization with a Polytron (Kinematica, Basel, Switzerland) (twice for 5 sec) in 2 ml of 10 mM Tris·HCl buffer, pH 7.4, containing 5 mM $MgSO_4$. Membranes were subsequently centrifuged at 50,000 × *g* for 15 min at 4°, and the resulting pellet was resuspended in assay buffer (50 mM Tris·HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, and 0.1% ascorbic acid) at 10 mg of wet weight/ml for D_2 and D_3 cells and 20 mg of wet weight/ml for D_4 cells. Incubations were carried out for 2 hr at room temperature in the presence of 0.05–1 nM [^{3}H]spiperone or 0.2 nM [^{3}H]spiperone for displacement studies and were initiated by the addition of 75 μl of membranes (50–100 μg of protein) in a final assay volume of 500 μl. The incubation was terminated by rapid filtration over GF/B filters presoaked in 0.3% PEI and washed twice with 10 ml of ice-cold 50 mM Tris·HCl, pH 7.4. Nonspecific binding was defined with 10 μM apomorphine, and radioactivity was determined by liquid scintillation counting in a Beckman Instruments (Palo Alto, CA) multipurpose scintillation counter.

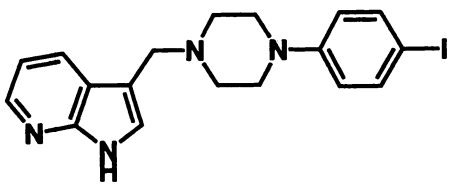

Fig. 1. Structure of L-750,667.

[^{125}I]L-750,667 binding assays. HEK cells expressing the human D_4 receptor were harvested and lysed, and membranes were prepared as described above. The final pellet was resuspended in assay buffer (50 mM Tris·HCl buffer, pH 7.4, containing 120 mM NaCl, 5.0 mM KCl, 5.0 mM $MgCl_2$, 1.5 mM $CaCl_2$ 5 mM EDTA, and 0.01% ascorbic acid) at 40 mg of wet weight/ml hD4 HEK cells. The kinetics of radioligand binding were determined before pharmacological characterization of [^{125}I]L-750,667 binding to hD4 HEK cells. For determination of the optimal association time, 0.02 nM [^{125}I]L-750,667 was incubated with 75 μl of membranes (30–80 μg of protein) in a final assay volume of 500 μl. The reaction was initiated by the addition of membranes, and the incubation was allowed to proceed for variable time periods (1–420 min). The incubation was terminated by rapid filtration over GF/B filters presoaked in 0.5% PEI and washed twice with 5 ml of ice-cold 50 mM Tris·HCl, pH 7.4. Nonspecific binding was defined with 1 μM haloperidol, and radioactivity was determined through counting in an LKB gamma counter. For dissociation experiments, 75 μl of membranes was incubated with 0.02 nM [^{125}I]L-750,667 in a final assay volume of 500 μl. Incubations were carried out for 4 hr at room temperature. Haloperidol (1 μM) was added to all tubes; the reaction was subsequently terminated at various time intervals (1–180 min) by rapid filtration, and radioactivity was determined as described above. For characterization of [^{125}I]L-750,667 binding to hD4 HEK cells, incubations were carried out for 4 hr at room temperature in the presence of 0.02–0.5 nM [^{125}I]L-750,667 or 0.03 nM [^{125}I]L-750,667 for displacement studies and initiated by the addition of 30–80 μg of protein in a final assay volume of 500 μl. The incubation was terminated as described earlier.

Adenylate cyclase. Confluent monolayers of hD4 HEK cells were grown in 24-well plates in DMEM containing 10% fetal calf serum. Cells were washed twice with 1 ml of serum-free DMEM containing 1 mM IBMX and then incubated with test compounds at 37° for 30 min in a final volume of 1 ml. In assays in which the effects of haloperidol and L-750,667 were studied, the cells were preincubated for 30 min in the presence of either haloperidol (1 μM) or L-750,667 (10 nM to 1 μM). Assays were terminated by washing the plates three times with 1 ml of ice-cold phosphate-buffered saline followed by the addition of 200 μl of ice-cold 5% trichloroacetic acid for 15 min. cAMP was extracted by addition of 3 × 1 ml of water-saturated diethyl ether followed by evaporation to dryness of 200 μl of the aqueous layer for 2–4 hr. Levels of cAMP were determined using the radioimmunoassay kit TRK 432 (Amersham International).

Statistical analysis. Binding parameters were determined by nonlinear least squares regression analysis using RS1 (BBN Research Systems, Cambridge, MA) and a computerized iterative procedure written by Dr. A. Richardson (Neuroscience Research Centre, Merck Sharp and Dohme Research Laboratories, Essex, UK). Statistical significance was determined using a pairwise *t* test analyzed with the use of BMDP software (BMDP Statistical Software, Los Angeles, CA). A value of $p < 0.05$ was considered statistically significant.

Results

Dopamine receptor selectivity of L-750,667. [^{3}H]Spiperone bound with high affinity to all three dopamine receptor cell lines (K_d: hD2 CHO, 0.04; hD3 HEK, 0.16; hD4

TABLE 1

Displacement of [^{3}H]spiperone binding to human D_2, D_3, and D_4 receptors by dopamine receptor agonists and antagonists

Results are expressed as the geometric mean of the apparent inhibition constant (K_i), with low and high standard errors given in parentheses. IC_{50} values were determined using a minimum of three separate experiments and were corrected to K_i values using the Cheng Prusoff equation (39). All competition curves were obtained from at least five to seven data points in each experiment.

Compound		hD2 CHO	hD3 HEK	hD4 HEK
Dopamine	K_i (nM)	560 (520, 610)	38 (25, 59)	17 (8.8, 33)
	n_H	0.8 ± 0.09	0.7 ± 0.1	0.6 ± 0.08
Quinpirole	K_i (nM)	410 (280, 600)	26 (23, 31)	34 (23, 49)
	n_H	0.8 ± 0.05	0.6 ± 0.06	0.7 ± 0.03
Haloperidol	K_i (nM)	1.4 (1.1, 1.8)	2.0 (0.95, 4.2)	2.3 (1.9, 2.9)
	n_H	1.0 ± 0.08	1.0 ± 0.05	0.9 ± 0.1
Clozapine	K_i (nM)	74 (65, 83)	200 (180, 220)	13 (10, 16)
	n_H	1.0 ± 0.05	0.9 ± 0.2	0.8 ± 0.09
L-750,667	K_i (nM)	>1700 (38%)	>4500 (38%)	0.51 (0.44, 0.58)
	n_H			1.3 ± 0.22

HEK, 0.11 nM). The binding capacity was 660 ± 110, 320 ± 70, and 230 ± 80 fmol/mg for hD2 CHO, hD3 HEK, and hD4 HEK cell lines, respectively. Dopamine receptor agonists such as dopamine and quinpirole showed high affinity displacement of [^{3}H]spiperone binding to D_2, D_3, and D_4 receptors (Table 1). Dopamine and quinpirole showed a 10–20-fold binding selectivity for the D_3 and D_4 receptor compared with the D_2 receptor. Neuroleptics also displayed high binding affinity, especially haloperidol, which displayed nanomolar affinity but no selectivity among the three receptors (Table 1). The putative D_4-selective antagonist clozapine had nanomolar affinity for all three dopamine receptors, although it had relatively small D_2/D_4 and D_3/D_4 selectivity (5.7- and 15-fold, respectively). The novel azaindole derivative L-750,667 (Fig. 1) displaced [^{3}H]spiperone binding to human D_4 receptors with subnanomolar affinity (K_i = 0.51 nM), but it had minimal activity at human D_2 and D_3 receptors. This corresponded to a human D_4 receptor selectivity of >2000-fold.

Receptor specificity of L-750,667. The azaindole L-750,667 was selective for D_4 receptors compared with its activity at other neurotransmitter receptors. No activity (IC_{50} >1 μM) was seen in a range of radioligand binding assays, including rat D_1/D_5 receptors labeled with [^{3}H]SCH23390 binding to rat striatal membranes (15), σ binding using [^{3}H]1,3-di-(2-[5-^{3}H]tolyl)-guanidine to guinea pig cortical membranes (modified from Ref. 16), and 5-hydroxytryptamine$_{1A}$ binding using [^{3}H]8-hydroxy-2-dipropylaminotetralin to pig cortex (17), and only with weak activity was seen in (IC_{50} = 950 nM) to 5-hydroxytryptamine$_2$ receptors labeled with [^{3}H]4-bromo-2,5-di-methoxyphenylisopropylamine-(±)-[*propyl*-1,2-^{3}H] binding in rat frontal cortex (18).

Initial characterization of [^{125}I]L-750,667 binding. No specific binding of [^{125}I]L-750,667 (0.03 nM) to membranes from hD2 CHO cells or hD3 HEK cells was observed under conditions that resulted in specific binding of the nonselective radioligand [^{3}H]spiperone. In contrast, [^{125}I]L-750,667 (0.02–0.5 nM) bound in a dose-dependent manner to hD4 HEK cell membranes. In the presence of either 1 μM haloperidol or 10 μM apomorphine, a specific binding window of ~85% was observed. In a typical experiment using 0.03 nM [^{125}I]L-750,667, the total binding was 7900 dpm, and the specific binding was 6600 dpm. Saturation studies revealed saturable binding that on Scatchard analysis revealed a single component with a dissociation constant (K_d) of 0.16 ± 0.06 nM and a binding capacity of 251 ± 71 fmol/mg of protein (Fig. 2A). The number of binding sites were similar to those observed with [^{3}H]spiperone (B_{max} = 227 ± 83 fmol/mg, K_d = 0.11 ± 0.01 nM; Fig. 2B) calculated using the same membrane

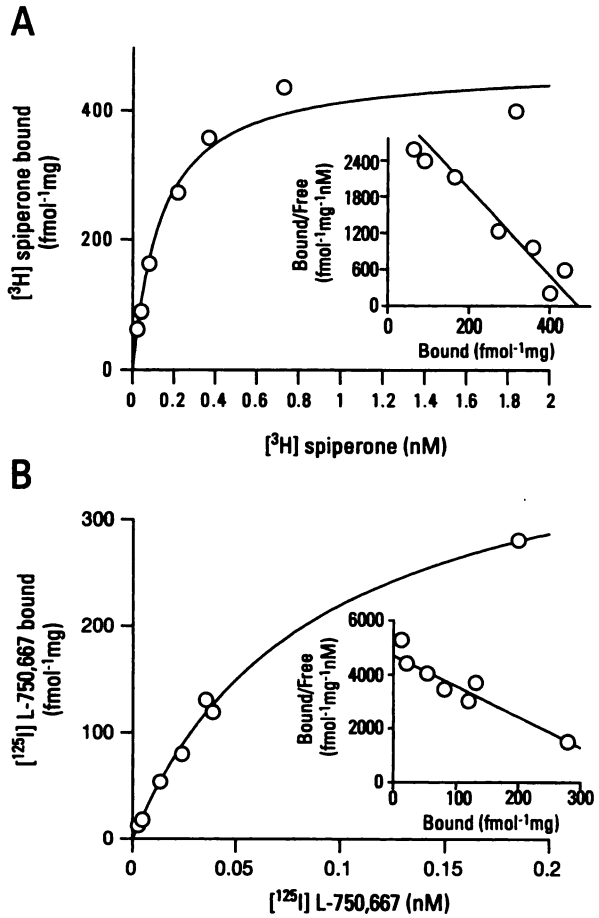

Fig. 2. Saturation of (A) [^{3}H]spiperone binding and (B) [^{125}I]L-750,667 binding to membranes from hD4 HEK cells. A, Specific binding (fmol/mg) of 0.05–1 nM [^{3}H]spiperone to hD4 HEK cell membranes. *Inset*, Scatchard transformation of the same data. K_d and B_{max} values were 0.14 nM and 470 fmol/mg, respectively. Results are representative from one of four separate experiments. B, Specific binding (fmol/mg) of 0.02–0.2 nM [^{125}I]L-750,667 to hD4 HEK cell membranes. *Inset*, Scatchard transformation of the specific binding data from the same experiment yielding K_d and B_{max} values of 0.09 nM and 410 fmol/mg, respectively. Results are representative from one of four separate experiments.

preparations, indicating that both radioligands are binding to the same number of sites, presumably the D_4 dopamine receptor.

Saturation studies were also performed on membrane preparations that contained either a 50:50 (mixture A) or an 80:20 (mixture B) mixture of hD2 CHO (10 ml/mg of wet weight) and hD4 HEK (20 ml/mg of wet weight) membranes, respectively. Both [^{3}H]spiperone and [^{125}I]L-750,667 bound specifically and saturably to these mixtures of cloned D_2 and D_4 receptors. Scatchard analysis of data from the [^{3}H]spiperone-binding studies best fit a single-component model with similar K_d values obtained in mixtures A and B (0.2 ± 0.01 and 0.22 ± 0.02 nM, respectively), whereas the B_{max} value increased in mixture B (523 ± 7 and 853 ± 19 fmol/mg in membrane mixtures A and B, respectively), presumably reflecting the higher density of D_2 receptors with the increase in the proportion of hD2 CHO membranes. In comparison, [^{125}I]L-750,667 bound to mixtures A and B with similar dissociation constants (0.21 ± 0.06 and 0.2 ± 0.02 nM, respectively), whereas the B_{max} values decreased in mixture B (412 ± 70 and 261 ± 13 fmol/mg in mixtures A and B, respectively). Because [^{3}H]spiperone binds to D_2 and D_4 receptors with similar dissociation constants, it was not possible to separate the D_4 binding component from mixed D_2/D_4 receptor preparations without using selective compounds to block either receptor subtype. However, the K_d values obtained for [^{125}I]L-750,667 in both membrane preparations correlate more closely with its binding affinity for D_4 than for D_2 receptors. Furthermore, the decrease in the binding capacity obtained in membrane preparation B probably reflects the decrease in hD4 HEK membranes, giving further support for a selective labeling of D_4 receptors with [^{125}I]L-750,667 with minimal binding to D_2 receptors, in a preparation in which there is a higher abundance of D_2 than D_4 receptors.

Binding kinetics of [^{125}I]L-750,667. The association of [^{125}I]L-750,667 to hD4 HEK membranes was relatively slow, reaching binding equilibrium at 4 hr (Fig. 3A). The dissociation of [^{125}I]L-750,667 binding by 1 μM haloperidol occurred over 2 hr (Fig. 3B). Calculation of the rate constants for these studies revealed an association constant (K_{on}) of 0.26 min^{-1} nM^{-1} (three experiments) and a dissociation constant (K_{off}) of 0.0048 ± 0.002 min^{-1} (three experiments). From the K_{on} and K_{off} values, the apparent equilibrium dissociation constant (K_{off}/K_{on}) was estimated to be 0.02 nM.

Pharmacology of [^{125}I]L-750,667 binding. The binding of 0.03 nM [^{125}I]L-750,667 to hD4 HEK cells was characterized with compounds previously shown to bind with high affinity to the D_4 receptor. Relatively high affinity was seen with a number of known neuroleptics, including haloperidol and chlorpromazine (Table 2). In contrast, the substituted benzamide sulpiride and the dopamine receptor antagonist (+)-butaclamol were relatively weak at inhibiting the binding of [^{125}I]L-750,667. These results are similar to those seen using [^{3}H]spiperone in this cell line and with previous results (7, 19, 20). Although the Hill coefficients for some of the antagonists are <1, the addition of 100 μM guanosine-5′-(β,γ-imido)triphosphate to the assay had no effect on the dose-response curves for these compounds (data not shown). The two enantiomers of sulpiride showed little stereoselective difference in the D_4 binding assay; however, (+)-butaclamol showed higher affinity for D_4 receptors compared with its (−)-enantiomer. The D_1 receptor antagonist (+)-SCH23390

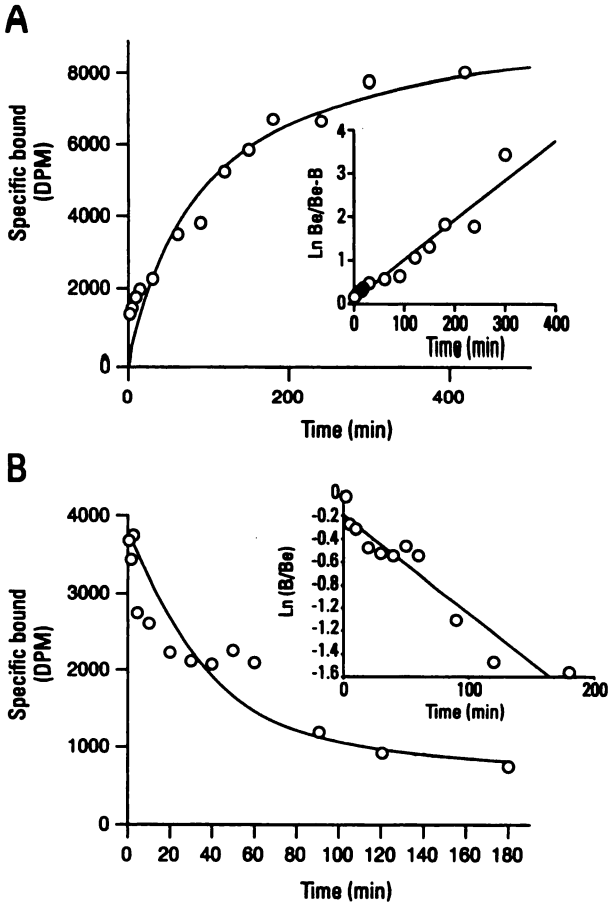

Fig. 3. Time course of (A) association and (B) dissociation of [^{125}I]L-750,667 binding to hD4 HEK cells. A, The association of [^{125}I]L-750,667 was performed by incubation of membranes with 0.03 nM [^{125}I]L-750,667 for various time points as described in Experimental Procedures. Nonspecific binding was determined with haloperidol (1 μM). Results are from one of three experiments, which produced quantitatively similar results; each experiment was performed in triplicate. *Inset*, data plotted as ln(Be/Be − B) versus time; K_{obs} = 0.01 ± 0.0004 (three experiments). B, The dissociation of binding was studied by incubating membranes with 0.03 nM [^{125}I]L-750,667 until equilibrium (4 hr), initiating the dissociation with the addition of haloperidol (1 μM) and terminating the reaction by filtration at various time points. Results are presented from one of three separate experiments; each experiment was performed in triplicate. *Inset*, data plotted as ln(B/Be) versus time after the initiation of dissociation; K_{-1} = 0.0048 ± 0.0021 (three experiments). *B*, amount of radioligand bound. *Be*, amount of ligand bound at equilibrium.

had weak activity at the D_4 receptor, confirming the selectivity of the binding assay. High binding affinity was also observed with a number of known dopamine receptor agonists, including dopamine, quinpirole, and the aminotetralin 6,7-ADTN (Table 2). 5,6-ADTN did not exhibit any D_4 binding affinity (K_i >8800 nM). The Hill coefficients for the dopamine receptor agonists do show a deviation from linearity, suggesting that agonists are recognizing high and low affinity components of [^{125}I]L-750,667 (Table 2) and [^{3}H]spiperone binding (Table 1). Based on the [^{125}I]L-750,667 binding studies, the dissociation constant of dopamine at the high affinity site was estimated as 5.3 ± 2.1 nM. This correlates well with the data obtained for dopamine in detailed displacement studies using [^{3}H]spiperone as the radioligand. In these latter studies, the K_d values for dopamine were 8 ± 3 nM at the high affinity site and 210 ± 90 nM at the low

TABLE 2

Pharmacology of [^{125}I]L-750,667 binding to hD4 HEK cells

Known dopamine receptor agonists and antagonists were evaluated for displacement of 0.03 nM [^{125}I]L-750,667 binding to hD4 HEK cells. Results are expressed as the geometric mean of the apparent inhibition constant (K_i) with low and high standard errors given in parentheses. IC_{50} values were determined on a minimum of three separate experiments and were corrected using the Cheng-Prusoff equation (39). All competition curves were obtained from at least five to seven data points in each experiment. Hill coefficient data (n_H) are presented as the arithmetic mean ± standard error. n denotes the individual numbers of replications.

Compound	K_i	n_H	n
	nM		
Dopamine	92 (65, 130)	0.50 ± 0.14	3
Quinpirole	100 (50, 200)	0.50 ± 0.13	3
6,7-ADTN	482 (270, 850)	0.58 ± 0.04	3
5,6-ADTN	>8800 (32%)		5
Haloperidol	2.2 (1.3, 3.7)	0.73 ± 0.06	4
Domperidone	90 (58, 139)	0.70 ± 0.06	5
(−)-Sulpiride	1900 (1500, 2500)	0.90 ± 0.11	4
(+)-Sulpiride	2200 (1600, 3100)	0.86 ± 0.12	5
Chlorpromazine	71 (51, 100)	0.86 ± 0.08	3
(+)-Butaclamol	550 (490, 610)	0.89 ± 0.10	7
(−)-Butaclamol	>8900 (13%)		4
(+)-SCH 23390	4600 (3000, 7000)	1.1 ± 0.11	3

affinity site, whereas the relative proportions of high and low affinity sites were 40% and 60%, respectively.

Functional studies with L-750,667. The linkage of activation of D_4 receptors with the inhibition of adenylate cyclase has been described elsewhere (21). Dopamine dose-dependently inhibited the stimulation of cAMP levels by the diterpine derivative forskolin (Fig. 4). The minimal effective dose for dopamine to inhibit forskolin (10 μM)-induced elevation of cAMP was 10 nM (9.9 ± 0.82–7.5 ± 0.51 pmol of cAMP/well). At the highest dose, dopamine (1 μM) produced a maximal reduction (40.7%) in the forskolin response (9.9 ± 0.82–5.9 ± 0.65 pmol of cAMP/well). The nonselective antagonist haloperidol at 1 μM completely reversed the inhibition of adenylate cyclase by 1 μM dopamine (data not shown). L-750,667 did not exhibit any intrinsic activity in this assay and did not inhibit the forskolin response at concentrations of ≤10 μM (data not shown). In contrast, L-750,667 (10 nM to 1 μM) dose-dependently reversed the inhibition of cAMP accumulation induced by 1 μM dopamine with a minimal effective dose of 10 nM and an ED_{50} value of 80 nM (Fig. 5). At the highest dose tested (10 μM), L-750,667 had no effect on the dopamine agonist-mediated inhibition of forskolin-induced elevation of cAMP in hD2 CHO or hD3 HEK cells (data not shown).

Discussion

The dopamine D_4 receptor shows nanomolar affinity for a range of neuroleptic drugs similar to that seen with D_2 and D_3 receptors. Interestingly, it exhibits relatively higher affinity for the atypical antipsychotic clozapine (7). Clozapine is used successfully in the clinic for the management of some treatment-resistant schizophrenics and exhibits a reduced propensity for extrapyramidal side effects. Although clozapine binds with high affinity to many other receptors (9), its affinity for the D_4 receptor has generated speculation that antagonism of D_4 receptors may be responsible for its antipsychotic activity. To validate this, one needs to identify compounds that exhibit high affinity and selectivity for the dopamine D_4 receptor subtype and to identify whether any changes in receptor affinity or number of D_4 receptors exist in the brain of schizophrenics.

The distribution of D_4 mRNA in the central nervous system has been studied using Northern blot analysis, polymerase chain reaction, and *in situ* hybridization techniques (7, 20, 22, 23). With the use of Northern blot analysis and polymerase chain reaction methods, relatively high levels of D_4 mRNA were localized in rat and primate midbrain, amygdala, frontal cortex, medulla, hypothalamus, and olfactory bulb (7, 20). *In situ* hybridization studies revealed a widespread distribution of D_4 mRNA; however, the distribution profile differs markedly from that of D_2 and D_3 mRNAs (22–25). Using both isotopically and nonisotopically labeled

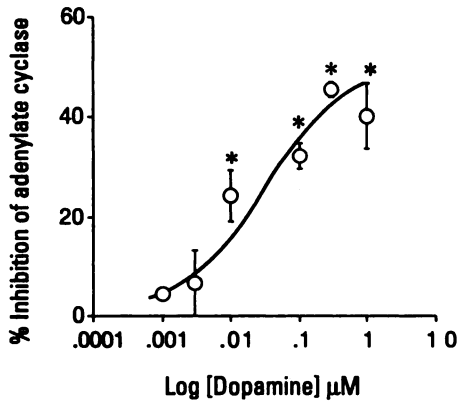

Fig. 4. Agonist inhibition of forskolin-stimulated cAMP levels in hD4 HEK cells. Levels of cAMP were determined using a radioimmunoassay described in Experimental Procedures. Basal cAMP levels were stimulated by 10 μM forskolin. The effect of dopamine (0.001–10 μM) was assessed on forskolin-elevated levels of cAMP. Results are expressed as percentage of inhibition of forskolin-induced increase in cAMP levels (mean ± standard error) and are from a typical experiment performed in triplicate. Each experiment was repeated at least three times with similar results. Statistical significance was determined using a pairwise *t* test using BMDP software. *, $p < 0.001$ compared with 10 μM forskolin alone.

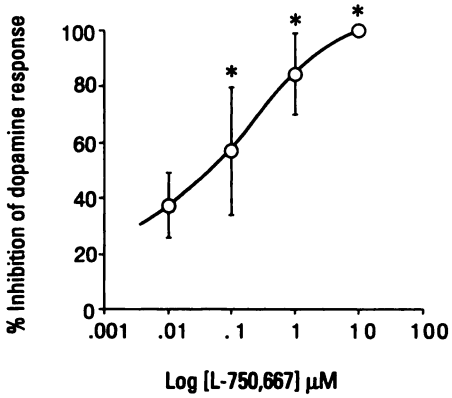

Fig. 5. Antagonism of dopamine induced inhibition of adenylate cyclase in hD4 HEK cells by L-750,667. Levels of cAMP were determined by radioimmunoassay as described in Experimental Procedures. Basal levels of cAMP were stimulated by the addition of the diterpine derivative forskolin (10 μM). Data are represented as the percentage of inhibition of dopamine (1 μM) response versus the logarithm of the concentration of L-750,667. The EC_{50} value for L-750,667 was calculated as the dose required to produce a 50% inhibition of the dopamine response. Results are from a typical experiment performed in triplicate; each experiment was repeated at least three times with similar results. *, $p < 0.001$ compared with forskolin (10 μM) in the presence of 1 μM dopamine.

D_4 probes, D_4 mRNA was localized in cortex, hippocampus, thalamus, hypothalamus, and substantia nigra in both rat and primate brain (20–22). The lower abundance of D_4 mRNA in the basal ganglia makes the D_4 receptor more appealing as a target for antipsychotic drug development because this may be related to the absence of motor side effects observed with clozapine therapy.

However, since the discovery of the D_4 receptor gene in 1991, little is known of the distribution of the D_4 receptor protein. The detection of mRNA indicates sites of receptor synthesis rather than sites of receptor localization, which may not be identical. Recently, Mrzljak *et al.* (26) reported, with the use of antibodies raised to a human D_4 receptor fusion protein, the presence of D_4-like immunoreactivity in GABAergic neurons in cortex, hippocampus, substantia nigra, and thalamus of the primate brain. It would be interesting to see whether this distribution corresponds to that observed with a selective D_4 receptor radioligand. Until now, there has been no report of selective dopamine D_4 receptor ligands. Using [^{3}H]emonapride and [^{3}H]raclopride as indirect measures of D_4 receptor density in the human postmortem brain, Seeman *et al.* (10) claimed that in the caudate of control human brain, the density of D_4 receptors was extremely low (<1 pmol/g of wet weight); however, in schizophrenic caudate, D_4 receptors were elevated by 6-fold (10). This was the first demonstration that D_4 receptors may be involved in the etiology of schizophrenia. However, recent studies revealed conflicting data either confirming (11, 12) or failing to prove (13) this claim. Indeed, Seeman *et al.* (27) quite rightly pointed out that their indirect methodology resulted in measurement of "D_4-like" receptors because they do not have the appropriate compounds with which to demonstrate whether this receptor population exhibits a D_4-like pharmacological profile.

In the current study, we identified an azaindole derivative, L-750,667, that displaces [^{3}H]spiperone binding to hD4 HEK cells with subnanomolar affinity and displays >2000-fold selectivity for D_4 receptors against other dopamine receptor subtypes. The dopamine receptor agonists dopamine and quinpirole displaced [^{3}H]spiperone from all three receptor subtypes at nanomolar concentrations with a 10–20-fold selectivity for D_3 and D_4 receptors, which is in agreement with previously published data (6, 7). The dose-response curves to dopamine and quinpirole showed low Hill coefficients (n_H = <0.8), which are not dissimilar to those seen with other G protein-linked receptors (28–30). This is thought to result from the ability of the agonist, receptor, and G protein to form a ternary complex. Of the dopamine receptor antagonists tested in this study, haloperidol showed high affinity and no selectivity among D_2, D_3, and D_4 receptors. This agrees with other published data (6, 7). We also demonstrated that L-750,667 has high affinity for D_4 receptors with little activity at human D_2 and D_3 receptors, rat D_1/D_5 receptors, σ binding sites, and 5-hydroxytryptamine$_{1A}$ or 5-hydroxytryptamine$_2$ receptors and therefore is a likely candidate for development as a D_4 radioligand.

The agonist activation of dopamine receptors in cell lines that express human D_2, D_3, and D_4 receptors has been reported to inhibit forskolin-stimulated cAMP accumulation (21, 31–33). In the current study, we have shown that activation of D_4 receptors by dopamine results in dose-dependent inhibition of cAMP accumulation in HEK cells. L-750,667 evaluated in this assay displayed antagonist activity by inhibiting dopamine-mediated inhibition of adenylate cyclase.

Because L-750,667 exhibited high affinity and selectivity for the dopamine D_4 receptor subtype, it was considered to be an ideal candidate for development as a radiolabel for the dopamine D_4 receptor. Radioiodine labeling was chosen in preference to tritium labeling of L-750,667 to obtain better detection for the low levels of D_4 receptor expression. Previous studies have demonstrated that iodinated radiolabels (e.g., [^{125}I]iodosulpride and [^{125}I]epidepride) enabled the detection of low levels of D_2 receptors in areas, such as the cortex, that were undetected by tritiated radioligands such as [^{3}H]spiperone and [^{3}H]raclopride (34–36). In the current study, we evaluated the suitability of [^{125}I]L-750,667 as a selective D_4 receptor radioligand.

Under optimal binding conditions, the displacement of [^{125}I]L-750,667 binding with a number of known dopamine receptor agonists and antagonists revealed that the pharmacology of the binding sites corresponded to the D_4 receptor, as shown by [^{3}H]spiperone binding to D_4 receptors in the current and a previous study (7). The stereoselectivity of binding seen with (+)-butaclamol and (−)-butaclamol and the lack of binding with (+)-SCH23390 confirms the specificity of D_4 receptor binding with [^{125}I]L-750,667. The two regioisomers (37) of the aminotetralin ADTN (6,7-ADTN and 5,6-ADTN) bound to D_4 receptors with differing affinities. The 6,7-isomer bound to D_4 receptors with an affinity of 480 nM and has previously been shown to be a potent dopamine receptor agonist (38). The weak D_4 affinity of the 5,6-isomer is also in agreement with the profile of these aminotetralins for other dopamine receptor subtypes (31).

In conclusion, we identified L-750,667 as one of the most selective high affinity D_4 receptor antagonists ever reported. Furthermore, we demonstrated that the iodinated radiolabel [^{125}I]L-750,667 is a subnanomolar ligand for the D_4 receptor, exhibiting an appropriate D_4 pharmacological profile in hD4 HEK cells. Results from the saturation studies on combinations of cloned D_2 and D_4 receptors revealed that the nonselective radioligand [^{3}H]spiperone cannot be used directly to discriminate between D_2 and D_4 receptor binding, whereas [^{125}I]L-750,667 was able to detect D_4 receptors in mixtures of cloned D_2 and D_4 receptors (a situation similar to that observed in native tissue) due to its high selectivity for D_4 receptors and minimal binding to D_2 receptors under the conditions used in this study. Studies are under way to directly measure the density of dopamine D_4 receptors in the brain with the use of [^{125}I]L-750,667.

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