Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 68 (2004) 761–772
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Comparative pharmacology of human dopamine D_2-like receptor stable cell lines coupled to calcium flux through $G\alpha_{qo5}$

Robert B. Moreland*, Masaki Nakane, Diana L. Donnelly-Roberts, Loan N. Miller, Renjie Chang, Marie E. Uchic, Marc A. Terranova, Earl J. Gubbins, Rosalind J. Helfrich, Marian T. Namovic, Odile F. El-Kouhen, Jeffrey N. Masters, Jorge D. Brioni

Neuroscience Research, Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL 60064, USA

Received 9 December 2003; accepted 11 May 2004

Abstract

The goal of this study was to develop a new approach to study the pharmacology of the dopamine D_4 receptor that could be used in comparative studies with dopamine D_2 and D_3 receptors. Stable HEK-293 cell lines co-expressing recombinant human D_{2L}, D_3 or D_4 receptors along with $G\alpha_{qo5}$ cDNA were prepared. Dopamine induced a robust, transient calcium signal in these cell lines with EC_{50}s for D_{2L}, D_3 and D_4 of 18.0, 11.9 and 2.2 nM, respectively. Reported D_4-selective agonists CP226269 and PD168077 were potent, partial D_4 agonists exhibiting 31–1700-fold selectivity for D_4 over D_3 or D_2. Non-selective D_2-like agonists apomorphine and quinpirole showed full efficacy but did not discriminate across the three receptors. D_3-selective agonists 7-hydroxy-DPAT and PD128907 were potent but non-selective D_2-like agonists. The reported D_3 partial agonist BP-897 exhibited minimal agonist activity at D_3 but was a potent D_3 antagonist and a partial D_4 agonist. Other D_2-like antagonists, haloperidol, clozapine, and domperidone showed concentration-dependent inhibition of dopamine responses at all three receptors with K_i ranging from 0.05 to 48.3 nM. The D_3 selective antagonist S33084 and D_4-selective antagonist L-745870 were highly selective for D_3 and D_4 receptors with K_b of 0.7 and 0.1 nM, respectively. Stable co-expression of D_2-like receptors with chimeric $G\alpha_{qo5}$ proteins in HEK-293 cells is an efficient method to study receptor activation in a common cellular background and an efficient method for direct comparison of ligand affinity and efficacy across human D_{2L}, D_3 and D_4 receptors.

Keywords: Dopamine; D_4 receptor; Calcium flux; Chimeric G-protein; $G\alpha_{qo5}$; A-369508

Abbreviations: A23187, 5-(methylamino)-2-(2*R*,3*R*,6*S*,8*S*,9*R*,11*R*)-3,9,11-trimethyl-8-[(1*S*)-1methyl-2-oxo-2-(1H)-pyrrol-2-yl-ethyl]-1,7-dioxaspiro[{5,5}undec-2-yl]methyl]-4-benzoxazolecarboxylic acid; ABT-724, 2-[(4-pyridin-2-ylpiperazin-1-yl)methyl]-1-H-benzimidazol; BP897, (*N*-[4-(4-(2-methoxyphenyl)piperazinyl)butyl]-2-naphthamide); CP226269, 5-fluoro-2-(4-pyridin-2-yl-piperazin-1-ylmethyl)-1H-indole; DPBS, Dulbecco's phosphate buffered saline; FLIPR, Fluorometric Imaging Plate Reader; $G\alpha_{qo5}$, Chimeric G-protein α-subunit; 7-hydroxy-DPAT, 7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin; L-741626, 4-(4-chlorophenyl)-1-(1H-pyrrolo[2,3-b]pyridin-3-ylmethyl)-piperidin-4-ol; L-745870, 3-[4-(4-chloro-phenyl)-piperazin-1-ylmethyl]-1H-pyrrolo[2,3-b]pyridine; PD128907, ((+)-(4a*R*,10b*R*)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-[1]benzopyrano[4,3-b]-1,4-oxazin-9-ol; PD168077, *N*-[4-(2-cyano-phenyl)-piperazin-1-ylmethyl]-3-methyl-benzamide; PIPAT, (*R*,*S*)-2′-trans-7-hydroxy-2-[*N*-*n*-propyl-*N*-(3′-iodo-2′-propenyl)-amino]tetralin; PNU95666E, 5-methylamino-5,6-dihydro-1H,4H-imidazo[4,5,1-ij]quinolin-2-one; PTX, pertussis toxin; $Ins(1,4,5)P_3$, Inositol 1,4,5-triphosphate; RIPA buffer, ristocetin-induced platelet aggregation buffer; S33084, biphenyl-4-carboxylic acid [4-(8-cyano-1,3a,4,9b-tetrahydro-3H-5-oxa-2-aza-cyclopenta[a]naphthalen-2-yl)-butyl]-amide; SCH23390, ((+)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol; SKF81297, *R*(+)-6-chloro-7,8-dihydro-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazephrine; TBST, Tris buffered saline with Tween 20

* Corresponding author. Tel.: +1 847 937 7920; fax: +1 847 935 1722.
E-mail address: robert.moreland@abbott.com (R.B. Moreland).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.05.019

1. Introduction

The actions of the neurotransmitter dopamine are mediated by specific G-protein coupled receptors which can be divided into two major families based on their ability to stimulate (D_1-like) or inhibit adenylate cyclase (D_2-like) [1,2]. Three human D_2-like receptors have been cloned: D_2, D_3 and D_4 [2,3]. The dopamine D_4 receptor is expressed predominantly within the central nervous system [4] and despite low abundance relative to the D_2 receptor, localization in cortex suggests an important functional role [5,6].

Recent studies have demonstrated that supraspinal, central nervous system dopamine D_4 receptors play a role in the regulation of penile erection as indicated by the pro-erectile effect of selective D_4 receptor agonists [7,8]. It has been suggested that selective D_4 agonists such as ABT-724 can be used to treat erectile dysfunction [8]. Activation of the dopamine D_4 receptor has also been proposed to play a role in cognition and attention deficit disorder [4]. One of

the challenges in this area is the development of an assay system for evaluation of D_4 agonists with selectivity against other D_2-like receptors.

Activation of the $G_{i/o}$-coupled dopamine D_4 receptor is most commonly reported as the inhibition of forskolin-stimulated cAMP accumulation [9] or the incorporation of radiolabeled GTP-γ-S into receptor-activated G-proteins [10]. Using GTP-γ-S incorporation, a comparative pharmacology of D_2-like receptors in the same cellular background with dopaminergic agonists and antagonists has been described [11]. Recently, the fluorometric imaging plate reader (FLIPR) was developed to perform rapid, functional characterization of agonists and antagonists with G-protein coupled receptor-transfected cells in a 96- or 384-well microplate format [12]. This method detects the increase in intracellular calcium resulting from activation of G_q-signaling pathways. Although activation of $G_{i/o}$-coupled receptors is not linked to calcium mobilization, co-expression of these receptors with chimeric $G\alpha_q$ proteins can facilitate this process. These recombinant, chimeric G-proteins contain the five carboxyl-terminal amino acids from $G\alpha_i$ or $G\alpha_o$, enabling G-protein binding to the receptor and concomitant signaling through G_q coupled pathways ($G\alpha_{qi5}$ or $G\alpha_{qo5}$) [12,13].

In this report, we describe the generation of stable cell lines in same host cellular background co-expressing the human dopamine $D_{4.4}$ receptor and chimeric G_{qo5} to generate a robust calcium response and facilitate detection using FLIPR. This approach was examined for the characterization of D_4 agonists and antagonists, and the subtype selectivity of these agents compared using stable cell lines co-expressing $G\alpha_{qo5}$ with either human D_{2L} or D_3 receptors.

2. Materials and methods

2.1. Cloning of human dopamine D_2-like receptors

The c-DNA clones for $G\alpha_{16}$ (in pCIneo, Promega) and $G\alpha_{qi5}$ (pCEP-G_{qi5}-HA, Molecular Dynamics) were purchased. The c-DNA clone for $G\alpha_{qo5}$ was generated from $G\alpha_{qi5}$ by site-specific mutagenesis.

The human $D_{4.4}$ receptor cDNA [14] was cloned from human retina polyA-containing RNA by PCR using the advantage GC polymerase mix with the addition of GC-Melt as described by the manufacturer (Clontech). Due to the high GC content of coding sequence, the PCR reactions also incorporated a 3:1 ratio of dGTP:7-deaza-dGTP as described [15]. The coding sequence was first amplified in overlapping amino terminal and carboxyterminal regions (overlapping a unique *Bsg*1 restriction endonuclease site) using the following oligonucleotide primers: amino terminal primer: humd4-5p-rs; 5′-CACTCGAGCCACCATGGGGAACCGCAGCACCGCGGAC and humd4-n3p; 5′-GCGGCCGTGCAGCTTG GCGCGAC. Carboxyterminal primers: humd4-c5p; 5′-CTGGGAGGTGGCACGTCGCGCCAAG and humd4-3p-rs; 5′-CATCTAGATCAGCAGCAGGCACGCAGGGCCTTG. Multiple PCR fragments were cloned and sequenced, followed by mutations correction by fragment replacement. The full-length coding sequence was constructed by ligation utilizing the unique *Bsg*1l restriction endonuclease site and cloned into the pCIneo vector (Promega). The final clone containing the complete coding sequence of the $hD_{4.4}$ allele was sequenced to confirm that additional mutations had not occurred during cloning.

As initial experiments to co-express human D_{2L} and different G-proteins were unsuccessful, the 5′ coding sequence of the chimeric $G\alpha_{qo5}$ protein gene was fused to the 3′ coding sequence of human D_{2L} gene such that the resulting fusion protein is predicted to encode D_{2L}: Ile, Leu, His, Cys; Linker Ser, Phe, Lys, Ser; and $G\alpha_{qo5}$: Met, Thr, Leu and so on through the protein sequence. The gene fusion was transfected into human HEK-293 cells as described below. This approach has been used to produce functional human D_{2S} in a baculovirus expression system [16]. A fragment coding for human dopamine D_2 receptor was generated using the polymerase chain reaction (PCR) with *Pfu* Turbo DNA polymerase (Stratagene) using as template pcEXV-D_2 (American Type Culture Collection) and kinased primers 5′-CACAGCTAGCCACCATGGATCCACTG and 5′-AAATGAGCAGTGGAGGATCTTCAGGAA, cut with restriction endonuclease *Nhe*I and gel-purified. A fragment coding for $G\alpha_{qo5}$ was generated by the PCR as described above using as template pCEP-G_{qo5}-HA (Molecular Devices) and kinased primers 5′-AAATCCATGACTCTGGAGTCCATCATGG and 5′-ACACGCGGCCGCTCAGTAGAGGCCACAGCCC, cut with restriction endonuclease *Not*I and gel-purified. The two fragments were ligated to *Nhe*I, *Not*I-digested, phosphatase treated pIRES-Neo2 (BD Biosciences Clontech) and transformed into DH5α competent cells (Invitrogen) and clones selected and identified.

The human D_3 receptor cDNA [17] was cloned by first reverse transcribing human brain cDNA total RNA (Clontech) with Superscript II reverse transcriptase using random hexamers (Invitrogen). The coding sequences for human D_3 were generated by nested PCR with *Pfu* Turbo DNA polymerase. The first PCR reaction used full-length D_3 primers 5′-CTATGGCATCTCTGAGTCAG and 5′-TCAGCAAGACAGGATCTTG AG. Material from this reaction was then re-amplified using the primer sets 5′-CACTCGAGCCACCATGGCATCTCTGAGTCAG, 5′-GTCACTCCAAAGGGCAGGT, 5′-CTTCTACCTGCCCTTTGGAG and 5′-CATCTAGATCAGCAAGACAGGATCTTGAG, which generated fragments coding for the N- or C-terminal portions respectively. DNA from individual clones was sequenced to identify clones without errors as compared to the published sequence. The N- and C-terminal fragments were prepared by digestion with restriction endonucleases *Xho*I + *Bsp*MI and *Not*I +

*Bsp*MI respectively, gel-purified, and ligated to *Xho*I, *Not*I digested expression vector pCIneo (Promega), transformed into DH5α competent cells (Invitrogen) and clones selected and identified.

2.2. Establishment of stable cell lines

DNA (7.5 μg/75 cm^2 flask) from sequenced clones for D_3 or $D_{4.4}$ were co-transfected using Lipofectamine 2000 as suggested by the manufacturer (Invitrogen) with pCEP-$G\alpha_{qo5}$-HA (7.5 μg/75 cm^2 flask) (Molecular Devices) into 90% confluent HEK-293 cells. Twenty four hours later, cells were split and stable colonies were selected with 200 mg/L Geneticin and 200 mg/L hygromycin B (Invitrogen). In some experiments, the same receptors were co-transfected with either $G\alpha_{16}$ (in pCIneo), or $G\alpha_{qi5}$ (pCEP-G_{qi5}-HA) and stably selected cell populations resistant to geneticin (200 μg/ml) and hygromycin (200 μg/ml) were assayed for calcium responses using FLIPR (described below). Stable cell lines were isolated by selecting for neomycin and hygromycin resistance. Individual clones were then screened for high activity based on calcium influx relative to the calcium ionophore A23187. For the $D_{2L}/G\alpha_{qo5}$ fusion, the plasmid described above (10 μg/75 cm^2 flask) was transfected into HEK-293 as described above only selecting for geneticin alone. Likewise, the cell line containing $G\alpha_{qo5}$ alone (10 μg/75 cm^2 flask) was transfected as described above and selected for hygromycin resistance colonies.

2.3. Western blot analysis of Gαq

Membranes from cells expressing D_3 and $D_{4.4}$ receptors were solubilized in SDS sample buffer in presence of proteases inhibitor cocktail (Roche Diagnostics), boiled for 15 min and loaded on NUPAGE Bis–Tris gels (4–12%) (Invitrogen). As a protein standard for $G\alpha_q$, purified recombinant rat $G\alpha_q$-His Tag (Calbiochem, molecular weight 44900 Da) was used for all determinations. Proteins were transferred onto PVDF membranes using a semidry blot apparatus (Biorad). The membranes were blocked with 10% nonfat dry milk in TBST wash buffer (25 nM Tris, 150 mM NaCl, 1% Tween 20, pH 7.6), and then incubated with rabbit anti-$G_{q/11a}$ specific antibody (Calbiochem) diluted 1:1000 in wash buffer containing 10% milk, overnight at 4 °C. The membranes were rinsed three times with wash buffer and then incubated with secondary horseradish peroxidase-conjugated anti rabbit IgG (1:5000, Amersham Biosciences). Membranes were rinsed three times with wash buffer and detection was performed using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech). Immunoblots were quantified by densitometric scanning of film exposed in the linear range using ImageQuant. Cells expressing $D_{2L}/G\alpha_{qo5}$ fusion protein were detached, washed with D-PBS and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, pH 8.0), in presence of protease inhibitor cocktail. Protein concentrations were determined using the BCA protein assay kit (Pierce). Samples were centrifuged to remove debris, and aliquots of supernatant were solubilized in SDS sample buffer in presence of proteases inhibitor cocktail. Electrophoresis and western blotting were performed as for D_3 and $D_{4.4}$ described above. B_{max} determinations for total receptor on each cell line were carried out using saturation with [^{3}H]-spiperone as previously described (see below) [18,20]. Protein concentrations were determined using the BCA protein assay kit (Pierce). G-proteins were quantified by scanning densitometry of know amounts (0.5–10 ng) of recombinant rat $G\alpha_q$ and the construction of a standard curve to estimate G-protein in the cell lines.

2.4. FLIPR assay

Cells were plated into 96-well, black-wall/clear-bottom microplates (Biocoat, Becton Dickinson) at 20,000 cells per well. After 2 days of culture, the culture medium was removed by aspiration and replaced by 0.1 ml of DPBS (Dulbecco's Phosphate Buffered Saline with D-glucose and sodium pyruvate) containing 0.04% Pluronic F-127 and 4 μM Fluo-4, fluorescent calcium indicator dye. After incubation for 1 h at room temperature, the cells were washed four times with DPBS in a plate washer (Molecular Devices). After the final wash, 150 μl of DPBS was added to each well. Fluorometric imaging plate reader (FLIPR384, Molecular Devices) transferred 50 μl from the compound plate to the cells and made fluorescence readings for 3 min (every second for the first minute and every 5 s for the next 2 min). All the data were normalized with the response of 10 μM dopamine.

2.5. cAMP determinations

Chinese hamster ovary cells expressing human $D_{4.4}$ (obtained from Dr. Hubert Van Tol, University of Toronto) were grown as reported [9]. 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% confluence. The cells were washed with D-PBS without Ca^{2+}/Mg^{2+} and harvested using a non-enzymatic cell dissociation buffer (Sigma–Aldrich). The cells were washed with 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 using

a nonlinear regression curve-fitting program (Prism, GraphPad Software).

2.6. Radioligand binding assays

Membranes were prepared from stable cell lines expressing either human D_{2L} or human D_3 in HEK-293 cells (gift of Dr. Liliane Unger, Abbott Laboratories, Ludwigshafen, Germany). HEK-293 transfected cells were cultured in RPMI supplemented with 10% fetal calf serum. Human dopamine $D_{4.4}$ receptor-transfected HEK-293 cells ($hD_{4.4}$-$G\alpha_{qo5}$ HEK were cultured in DMEM supplemented with 10% fetal calf serum, 1 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). For membrane preparation, the cells were seeded into a Cell Factory (VWR) and the confluent cells were detached with cell dissociation buffer (Invitrogen). The cell pellet was homogenized using a Polytron for 10 s in 50 mM Tris–HCl, pH 7.4 and centrifuged at 30 min for 100,000 × *g*. Membrane aliquots were stored at −80 °C until use.

For human D_{2L}, binding assays were initiated by addition 250 μl of membrane to 200 μl of [^{125}I]-PIPAT (Amersham Pharmacia Biotech) and incubated at room temperature for 1 h [18,19]. The incubation buffer consisted of 0.6 nM [^{125}I]-PIPAT, 50 mM Tris–HCl, pH 7.4, 10 mM $MgSO_4$, and 1 mM EDTA. Nonspecific binding was determined in the presence of 10 μM spiperone. The reaction was terminated by filtration using a Filtermate Harvester (Packard). Radioactivity was measured by TopCount Microplate Scintillation Counter (Packard). In experiments to determine the B_{max} of the $D_{2L}/G\alpha_{qo5}$ cell line, saturation binding assays were conducted with 20 μg $hD_{2L}/G\alpha_{qo5}$ membranes using 0.01–16 nM [^{3}H]-spiperone (Amersham) in 50 mM Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM Mg Cl_2, 2 mM $CaCl_2$ [18] Nonspecific binding was determined in the presence of 10 μM haloperidol. Data were analyzed by nonlinear regression using curve-fitting program (Prism, GraphPad Software).

Human D_3 binding assays were initiated by addition 250 μl of membrane to 200 μl of [^{3}H]-7-OH-DPAT (Amersham) and were incubated at room temperature for 1h. Nonspecific binding was determined in the presence of 10 μM spiperone. The incubation buffer consisted of 50 mM Tris–HCl, pH 7.4, 10 mM $MgSO_4$, and 1 mM EDTA. In competition binding studies, agonists or antagonists were prepared with 0.1% ascorbic acid in the buffer. The final concentration for [^{3}H]-7-OH-DPAT was 0.6 nM. The reaction was terminated by rapid filtration through UniFilter-96 GF/B filers, using a Filtermate Harvester. Filters were washed three times with 1 ml of ice cold 50 mM Tris–HCl, pH 7.4. Radioactivity was determined using a TopCount Microplate Scintillation Counter (Packard). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce) with BSA as a standard. In experiments to determine the B_{max} of the $D_3/G\alpha_{qo5}$ cell line, saturation binding assays were conducted as described above for the D_2 cell line.

Human $D_{4.4}$ binding assays were initiated by addition 250 μl of membrane to 200 μl of [^{3}H]-A-369508 and were incubated at room temperature for 1 h [20]. Nonspecific binding was determined in the presence of 10 μM PD168077 (Tocris). The incubation buffer consisted of 50 mM Tris–HCl, pH 7.4, 5 mM KCl, 120 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA. In competition binding studies, agonists or antagonists were prepared with 0.1% ascorbic acid in the buffer. The final concentration for [^{3}H]-A-369508 was 2 nM. The reaction was terminated by rapid filtration through UniFilter-96 GF/B filers, using a Filtermate Harvester (Packard). Filters were washed three times with 1 ml of ice cold 50 mM Tris–HCl, pH 7.4. Radioactivity was measured by TopCount Microplate Scintillation Counter (Packard). Proteins were determined by BCA Protein Assay Kit (Pierce) using BSA as a standard. Competition curves for nonradioactive compounds were analyzed by nonlinear regression using curve-fitting program (Prism, GraphPad Software). All assays were performed in triplicate and IC_{50} values were converted to K_i values by the method of Cheng and Prusoff [21]. In experiments to determine the B_{max} of the $D_{4.4}/G\alpha_{qo5}$ cell line, saturation binding assays were conducted as described above for the D_2 cell line except the incubation buffer included 50 mM Tris–HCl, pH 7.4, 5 mM KCl, 120 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA and samples were incubated at room temperature for 2 h [9,20].

2.7. Materials

Fluo-4 and Pluronic F-127 were purchased from Molecular Probes. DPBS, G418, hygromycin B and tissue culture reagents were from Invitrogen/Life Technologies. CP226269 [22], PD168077 [23], PNU95666E [24], BP897 [25] and S33084 [26] were prepared at Abbott Laboratories. All other chemicals were purchased from Sigma unless otherwise noted.

3. Results

3.1. Characterization of cell lines

In order to establish stable cell lines for comparison of D_2-like receptor pharmacology in the same cellular background, chimeric G-proteins were co-transfected with the receptors in HEK-293 cells and the maximum calcium response to dopamine determined for $G\alpha_{16}$, $G\alpha_{qi5}$ or $G\alpha_{qo5}$. For human D_3 and $D_{4.4}$ receptors, only $G\alpha_{qo5}$ gave response to dopamine (data not shown). All G-protein combinations with human D_{2L} were ineffective. Given the recent reported success of a human D_{2S} in an assay fusing $G\alpha_o$ [16], a gene fusion of the $G\alpha_{qo5}$ to the carboxy-terminus of the human D_{2L} receptor was prepared that

Table 1
B_{max} of cell lines and quanitation of G-proteins

Cell line	B_{max} (fmol/mg)	$G\alpha_q$ (ng/mg)	$G\alpha_q$ (fmol/mg)	Adjusted ratio
$G\alpha_{qo5}$	NA	7.76 ± 2.64	59.4	NA
$D_{2L}/G\alpha_{qo5}$	1542 ± 202	0.53 ± 0.04	5.3	1.0
$D_3/G\alpha_{qo5}$	1429 ± 174	0.67 ± 0.24	15.0	3.0
$D_{4.4}/G\alpha_{qo5}$	1030 ± 19	1.36 ± 0.47	30.7	8.6

B_{max} was determined for total D_2-like receptor expression using [^{3}H]-spiperone [18,20] and is expressed as fmol/mg membrane protein. $G\alpha_q$ was determined using SDS-PAGE analysis and protein standards (Fig. 1) followed by scanning densitometry. Samples were normalized per mg protein of extract. $G\alpha_q$ was estimated in extracts using the following molecular weights – $G\alpha_{qo5}$: 43000 Da; $D_{2L}/G\alpha_{qo5}$ fusion: 100,000 Da. NA-not applicable. Data are the average of three determinations. The adjusted ratio assumes that the ratio of $D_{2L}/G\alpha_q$ is 1:1 as a protein fusion, that the measurement is comparable across the receptors and reflects the final ratio. All measurements $n = 3$.

allowed stable expression of D_{2L} with a robust signal in the presence of dopamine. In any transfected cell line, the ratio of receptor to G-protein may be a factor in signal transduction. Receptor/G-protein fusions optimize this ratio at 1:1 [16]. The B_{max} for the three D_2-like cell lines determined using [^{3}H]-spiperone ranged from 1050–1400 fmol/mg (Table 1). Western blots using $G\alpha_q$ standards are shown

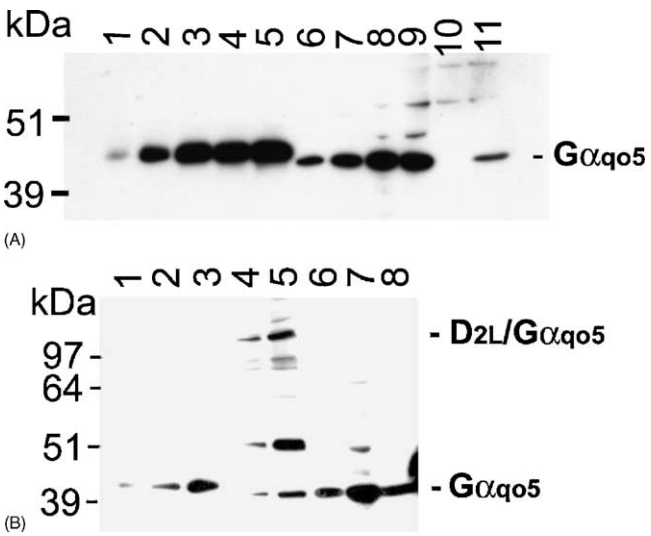

Fig. 1. Western blot analysis of $G\alpha_q$ in HEK cell lines. Panel (A): 4–20% gradient NUPAGE Bis–Tris polyacrylamide gel of G-proteins from cell lines. Lanes 1–5, rat recombinant Gαq (0.5, 1.5, 3, 4.5, and 6 ng/lane, respectively); lanes 6–7, human $D_3/G\alpha_{q05}$ cell line (2.5 and 5 ng/lane, respectively); lanes 8–9, human $D_{4.4}/G\alpha_{q05}$ cell line (2.5 and 5 ng/lane, respectively); lane 10, HEK-293 untransfected (1 μg/lane); and lane 11, HEK-293 $G\alpha_{q05}$ cell line (0.5 ng/lane). The $G\alpha_q$ reference band is 44900 Da and the corresponding band in the cell lines is ~43000 Da. Panel (B): 4–20% gradient NUPAGE Bis–Tris polyacrylamide gel of human $D_{2L}/G\alpha_{q05}$ fusion cell line. Lanes 1–3, human recombinant Gαq (3, 6, and 9 ng/lane, respectively); lanes 4–5, human $D_{2L}/G\alpha_{q05}$ fusion cell line (5 and 10 ng/lane, respectively); lane 6, human $D_3/G\alpha_{q05}$ cell line (5 ng/lane); lane 7, human $D_{4.4}/G\alpha_{q05}$ cell line (5 ng/lane); and lane 8, HEK-293 $G\alpha_{q05}$ cell line (0.5 ng/lane). The $G\alpha_q$ reference band is 44900 Da and the corresponding band in the $D_{2L}/G\alpha_{q05}$ fusion cell line cell lines is ~100,000 Da.

in Fig. 1. For the $G\alpha_{qo5}$ alone, D_3 and $D_{4.4}$ cell lines, $G\alpha_{qo5}$ was visualized as a ~43000 Da protein (Fig. 1A). The intact $D_{2L}/G\alpha_{qo5}$ fusion protein could only be visualized by extracting the cells first in RIPA buffer (Fig. 1B). The fusion is an ~100,000 Da protein that is immunoreactive with $G\alpha_q$ antibody. Only the intact fusion was quantified for measurements in Table 1. Assuming that the fusion has a ratio of 1:1 G-protein to receptor and that this is an accurate measurement, the ratio of G-protein/receptor for D_3 and $D_{4.4}$ cell lines were 3.0:1 and 8.6:1, respectively. The difficulty in visualizing the D_2 fusion protein may result in overestimates the G-protein ratio in the other two cell lines. Nevertheless, in all three cell lines, G-protein is not limiting.

Dopamine induced a concentration-dependent robust calcium signal in the human $D_{2L}/G\alpha_{qo5}$ HEK-293 cell line (Fig. 2, Panel A). The increase in intracellular calcium was transient and reached maximum levels within 20 s of compound administration and decayed to baseline within 3 min, typical for other GPCR responses mediated by $G\alpha_q$ [12]. Dopamine induced similar concentration-dependent increases in intracellular calcium in the human $D_3/G\alpha_{qo5}$ HEK-293 cell line (Fig. 2, Panel B) and human $D_{4.4}/G\alpha_{qo5}$ HEK-293 cell line (Fig. 2, Panel C) with EC_{50s} for dopamine of 18.0 ± 2.0, 11.9 ± 0.9 and 2.2 ± 0.2 nM for D_{2L}, D_3 and $D_{4.4}$, respectively. This activity was inhibited by the dopaminergic receptor antagonists haloperidol, domperidone and clozapine (see below). The response of human $D_{4.4}$ was slightly faster than D_2 or D_3 (peak response at 12 s versus 17 and 20 s) (Fig. 2). There was no effect of dopamine on calcium signaling in HEK293 untransfected cells or HEK293 stably transfected with $G\alpha_{qo5}$ alone (data not shown).

The mechanism of agonist-evoked increase of intracellular calcium was examined for all three receptors and found to be consistent with G_q signal transduction. Pre-incubation for 5 min with U-73122 (phospholipase C inhibitor; $IC_{50} = 3.1 \pm 0.3$, 6.9 ± 1.0 and 7.5 ± 1.9 μM for D_{2L}, D_3 and $D_{4.4}$, respectively) or 2-aminoethoxydiphenyl borate (inositol-triphosphate-induced Ca^{2+} release inhibitor; $IC_{50} = 3.4 \pm 0.8$, 22.1 ± 5.0 and 10.0 ± 1.0 μM for D_{2L}, D_3 and $D_{4.4}$, respectively) abolished calcium flux in response to dopamine. Pre-incubation for 5 min with reticular Ca^{2+}-ATPase inhibitors thapsigargin ($IC_{50} = 162 \pm 46$, 76.4 ± 23.0 and 41.7 ± 3.0 nM for D_{2L}, D_3 and $D_{4.4}$, respectively) and cyclopiazonic acid ($IC_{50} = 144.3 \pm 17.0$, 105.5 ± 12.0 and 119.4 ± 6.0 nM for D_{2L}, D_3 and $D_{4.4}$, respectively) also abolished calcium flux.

3.2. Pharmacology of $D_{4.4}$ cell line

Agonists and antagonists were assayed in a cell line stably expressing the human $D_{4.4}$ receptor. Reported D_4 agonists PD168077 and CP226269 were potent, partial agonists inducing calcium flux ($EC_{50} = 5.6$ and 32.0 nM and 68 and 46% of 10 μM dopamine, respectively).

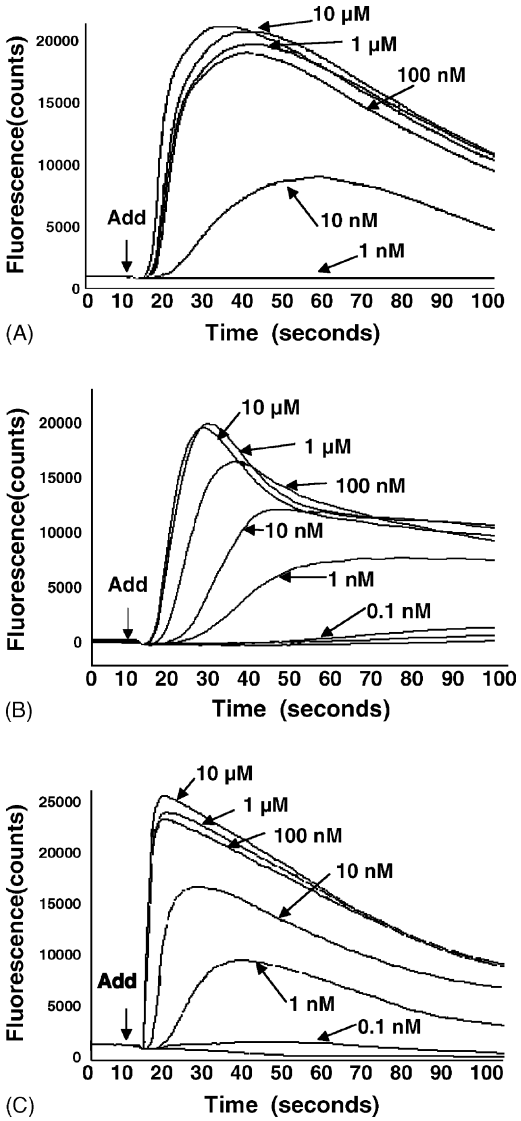

Fig. 2. Increase in intracellular calcium as a result of increasing dopamine concentration as measured by fluorescence in D_2-like receptor G_{qo5} HEK-293 cell lines. Dopamine was added in a concentration-dependent fashion as described in experimental procedures. Panel (A): representative tracing from D_{2L}-G_{qo5} HEK-293 cells. Panel (B): representative tracing from D_3-G_{qo5} HEK-293 cells. Panel (C): representative tracing from $D_{4.4}$-G_{qo5} HEK-293 cells. ADD equals the point of dopamine addition.

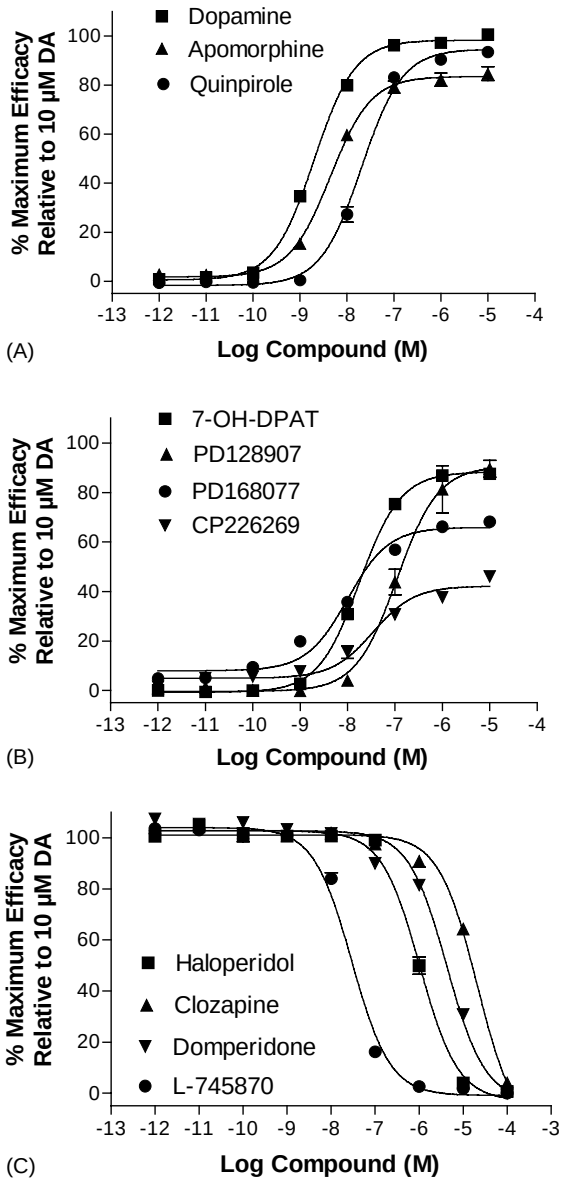

Fig. 3. Effects of dopamine agonists and antagonists on $D_{4.4}$-HEK cell line. Panel (A): effects of D_2-like agonists dopamine (closed squares), apomorphine (closed triangles) and quinpirole (closed circles). Panel (B): effects of D_3 agonists 7-OH-DPAT (closed squares), and PD128907 (closed triangles) and D_4 agonists PD168077 (closed circles) and CP226269 (closed inverted triangles). Panel (C): effects of antagonists against 1 µM dopamine. The cells were pre-incubated with various concentrations of haloperidol (closed squares), clozapine (closed triangles), domperidone (closed inverted triangles) and L-745870 (closed circles) for 5 min at room temperature, add 1 µM of dopamine to measure the calcium response by the FLIPR. Data points represent mean ± S.E.M. $n = 4$.

Compounds assayed included compounds selective for other dopaminergic subtypes as well as D_2-like agonists. The D_1-selective agonist SKF81297 and the reported D_2-selective agonist PNU-95666E were inactive in this assay as well as in D_4 receptor competition binding assays. Reported D_3-selective agonists 7-hydroxy-DPAT and PD128907 were both potent and efficacious at D_4 (Fig. 3, Table 2). Reported partial D_3 agonist BP-897 [25] had partial agonist activity at the D_4 receptor in this assay (Table 2). Only quinpirole had maximal responses comparable to the dopamine response ($EC_{50} = 20.6$ nM and 94% of 10 µM dopamine) while the remaining agonists resulted in lower maximal responses suggesting partial agonist responses for the D_4 receptor. PD 128907 and 7-hydroxy-DPAT were next most efficacious followed by apomorphine and reported D_4-selective agonists PD168077 and then CP226269. The rank order potency in this assay was dopamine > apomorphine, PD168077 > 7-hydroxy-DPAT, quinpirole > PD128907 > BP-897. A competitive binding assay using a D_4-selective agonist radioligand [^{3}H]-A-369508 has been developed in our lab [20] and complementary data for each of the agonists described above are shown in Table 2. Both SKF81297 and

Table 2
Characterization of dopamine agonists and antagonists in $D_{4.4}$ receptor cell line

Compound	Human $D_{4.4}$ FLIPR		Human $D_{4.4}$ binding, [^{3}H]-A-369508 K_i (nM)
	EC_{50} (nM)	Efficacy (%)	
Dopamine	2.2 ± 0.2	100	56.2 ± 9.1
Apomorphine	4.3 ± 0.2	84	4.0 ± 0.5
Quinpirole	20.6 ± 1.3	94	91.0 ± 10.0
SKF81297	>10000	0	>10000
PNU-95666E	>10000	7	>10000
PD128907	111.0 ± 5.0	89	1040.0 ± 63.0
7-OH-DPAT	18.0 ± 0.3	88	393.0 ± 69.4
BP-897	485.5 ± 93.9	66	277.3 ± 86.5
PD168077	5.6 ± 0.7	68	11.9 ± 1.1
CP226269	32.0 ± 7.0	46	0.8 ± 0.1
L-745870	2230 ± 1010	20	0.4 ± 0.01
	IC_{50} (nM)	K_b (nM)	[^{3}H]-A-369508 K_i (nM)
Haloperidol	4802.0 ± 549.0	10.5	1.9 ± 0.3
Domperidone	14100	31.0	30.4 ± 4.7
Clozapine	1121.0 ± 80.0	2.5	27.9 ± 1.8
SCH23390	>10000	–	>10000
L-741626	>10000	–	551.0 ± 94.6
S-33084	>10000	–	>10000
L-745870	42.0 ± 7.0	0.1	0.4 ± 0.01

Agonist activity was assayed by concentration response curves of various compounds with different reported selectivities for $D_{4.4}$ and percent efficacy normalized to 10 μM dopamine. IC_{50}s determined in the presence of 1 μM dopamine. K_bs were calculated by the method of Cheng–Prusoff [21]. These studies were complemented with binding using the D_4-selective agonist radioligand [^{3}H]-A-369508 [20]. All determinations represent the mean ± S.E.M., $n = 4$.

PNU-95666E that do not activate the receptor in calcium flux assays do not compete in the binding assay. While all of the compounds with agonist activity in calcium flux assays do bind to the receptor, there are differences in the rank order potencies.

Dopamine induced calcium flux in the $D_{4.4}/G_{qo5}$ cell line was antagonized by both D_2-like and D_4-selective dopamine receptor antagonists in a concentration-dependent manner (Fig. 3B, Table 2), while the D_1-selective and D_2 selective antagonists (SCH23390 and L-741626 respectively) had no effect. None of the antagonists tested with the exception of L-745870 elicited a calcium response on their own (data not shown). L-745870 at the highest concentration elicited a weak, partial agonist response ($EC_{50} = 2230$ nM, 20%) (Table 2). In contrast, when assayed against dopamine, the D_4-selective antagonist L-745870 gave the most potent antagonist response of the compounds tested. The Cheng–Prusoff method [21] was used to estimate K_b values which give an antagonist rank order L-745870 (0.1 nM) > clozapine (2.5 nM) > haloperidol (10.5 nM) > domperidone (43.9 nM). When these compounds were examined in competition binding assays with [^{3}H]-A-369508, binding correlated with antagonist activity. The one exception was the reported D_2-selective antagonist L-741626 which showed binding (551 nM) but did not inhibit dopamine-induced agonist responses in the cell line.

3.3. Pharmacology of D_{2L} cell line

When examined in the D_{2L} cell line, the D_2-like agonists apomorphine and quinpirole as well as the reported D_2-selective agonist PNU-95666E [24] showed potent agonist activities (86–92% efficacy in comparison to 10 μM dopamine) with 5.8, 40.0 and 190.0 nM EC_{50}, respectively (Fig. 4, Table 3). For all compounds these potencies are comparable to competition binding with [^{125}I]-PIPAT on human D_{2L} expressed in HEK-293 cells (Table 3). The D_1-selective agonist SKF81297 showed no functional activity but did compete in binding assays with [^{125}I]-PIPAT. Reported D_3 agonists 7-hydroxy-DPAT and PD128907 showed potent D_2 agonist activities in this assay (Table 3). These potencies are also comparable to competition binding with [^{125}I]-PIPAT on native human D_{2L} (Table 3) and together confirm that reported D_3-selective agonists 7-hydroxy-DPAT and PD-128907 are potent agonists on the cloned D_{2L} receptor. D_3 partial agonist BP-897 showed no agonist activity at D_2 despite potent binding ($K_i = 0.2$ nM). D_4-selective agonists CP226269 and PD168077 had no detectable agonist activity on D_{2L} despite competitive binding with PIPAT on D_{2L} membranes (120.0 and 1050.0 nM, respectively).

Table 3
Characterization of dopamine agonists and antagonists at D_{2L} receptor cell line

Compound	Human D_{2L} FLIPR		Human D_{2L} binding, [^{125}I]-PIPAT K_i (nM)
	EC_{50} (nM)	Efficacy (%)	
Dopamine	18.0 ± 2.0	100	10.0 ± 1.5
Apomorphine	5.8 ± 0.3	86	3.6 ± 0.4
Quinpirole	40.0 ± 4.0	90	38.4 ± 6.1
SKF81297	>10000	0	208.0 ± 2.3
PNU-95666E	190.0 ± 17.0	92	53.8 ± 4.2
7-OH-DPAT	6.1 ± 0.6	84	8.3 ± 0.5
PD128907	22.0 ± 3.0	95	15.1 ± 1.3
BP-897	>10000	0	0.2 ± 0.04
PD168077	>10000	0	1050.0 ± 72.6
CP226269	>10000	4	120.0 ± 9.0
	IC_{50} (nM)	K_b (nM)	[^{125}I]-PIPAT K_i (nM)
Haloperidol	2.6 ± 0.2	0.05	0.3 ± 0.04
Domperidone	10.3 ± 0.9	0.2	1.2 ± 0.08
Clozapine	223.0 ± 20.0	3.9	34.8 ± 2.8
SCH23390	>10000	–	>10000
L-741626	566.0 ± 188.0	9.8	22.9 ± 2.3
S-33084	>10000	–	338.0 ± 1.2
L-745870	>10000	–	>10000

Agonist activity was assayed by concentration response curves of various compounds with different reported selectivities for D_{2L} and percent efficacy normalized to 10 μM dopamine. IC_{50}s determined in the presence of 1 μM dopamine. K_bs were calculated by the method of Cheng–Prusoff [21]. These studies were complemented with binding using the D_2-like agonist radioligand [^{125}I]-PIPAT. All determinations represent the mean ± S.E.M., $n = 4$.

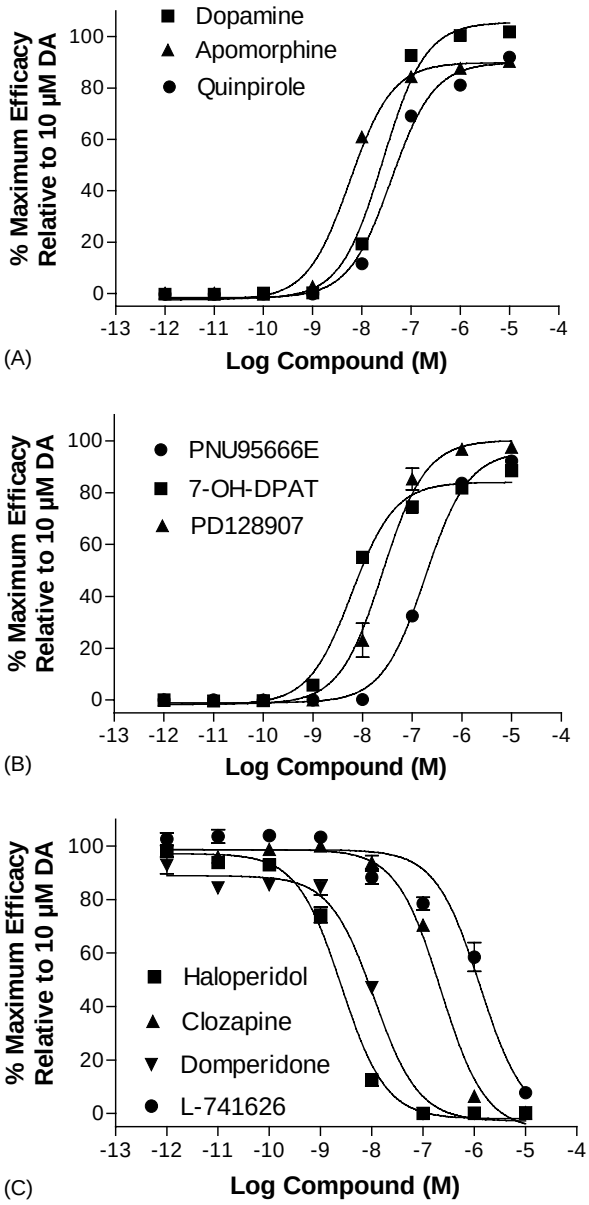

Fig. 4. Effects of dopamine agonists and antagonists on D_{2L}-HEK cell line. Panel (A): effects of D_2-like agonists dopamine (closed squares), apomorphine (closed triangles) and quinpirole (closed circles). Panel (B): effects of D_2 agonists PNU95666E (closed circles), and D_3 agonists 7-OH-DPAT (closed squares), and PD128907 (closed triangles). Panel (C): effects of antagonists against 1 μM dopamine. The cells were pre-incubated with various concentrations of haloperidol (closed squares), clozapine (closed triangles), domperidone (closed inverted triangles) and L-741626 (closed circles) for 5 min at room temperature, add 1 μM of dopamine to measure the calcium response by the FLIPR. Data points represent mean ± S.E.M. $n = 4$.

Both D_2-like and D_2-selective dopamine receptor antagonists antagonized dopamine-induced calcium flux in the D_{2L}/G_{qo5} cell line in a concentration-dependent manner (Fig. 4, Table 3). None of the compounds tested induced any calcium flux by on their own (data not shown) indicating that the compounds have no D_2 agonist activity in this assay. The D_2-like antagonists, haloperidol and domperidone showed concentration-dependent inhibition of dopamine response at D_2 with calculated K_bs of 0.05 and 0.2 nM, respectively (Table 3). The calculated K_bs were six-fold more potent than K_i determined by competition binding with [^{125}I]-PIPAT on native human D_{2L} (Table 3). Reported D_2 selective antagonist L-741626 blocked the effects of 1 μM dopamine (600 nM IC_{50}) in D_2 with a calculated K_b (9.8 nM) twice as potent as the K_i determined by competition binding with [^{125}I]-PIPAT on native human D_{2L} (23 nM K_i) (Table 2). Clozapine had potent activity on D_2 as well ($K_b = 3.9$ nM, K_i by competition binding = 39 nM, Table 2). Rank order potencies were haloperidol > domperidone > clozapine > L-741626. This was in contrast to the D_1-selective antagonist SCH23390, D_3 selective antagonist S33084 and the D_4-selective antagonist L-745870 that did not inhibit the 1 μM dopamine response at D_{2L}. Of these three antagonists, only S33084 exhibited binding on D_{2L} in competition binding assays with radiolabeled PIPAT.

3.4. Pharmacology of D_3 cell line

Agonists and antagonists were assayed in a cell line stably expressing the human D_3 receptor. Non-selective D_2-like agonists, apomorphine and quinpirole showed good efficacies and potencies at D_3 cell line, comparable to competition binding with [^{3}H]-7-hydroxy-DPAT on human D_3 membranes (Fig. 5, Table 4). On the other hand,

Table 4
Characterization of dopamine agonists and antagonists at D_3 receptor cell line

Compound	Human D_3 FLIPR		Human D_3 binding,
	EC_{50} (nM)	Efficacy (%)	[^{3}H]-DPAT K_i (nM)
Dopamine	11.9 ± 0.9	100	6.0 ± 1.2
Apomorphine	14.2 ± 2.0	80	5.0 ± 0.3
Quinpirole	17.3 ± 3.3	90	4.5 ± 0.6
SKF81297	1090.0 ± 131.0	49	260.0 ± 11.9
PNU-95666E	62.2 ± 10.3	79	412.0 ± 33.0
7-OH-DPAT	13.5 ± 1.2	82	0.6 ± 0.01
PD128907	29.7 ± 2.6	81	1.1 ± 0.1
BP-897	193.8 ± 36.7	16	0.4 ± 0.05
PD168077	>10000	5	2540.0 ± 396.0
CP226269	985.0 ± 245.0	55	521.0 ± 39.0
	IC_{50} (nM)	K_b (nM)	[^{3}H]-DPAT K_i (nM)
Haloperidol	4.4 ± 0.1	0.1	3.6 ± 0.3
Domperidone	7.2 ± 0.8	0.1	5.4 ± 0.2
Clozapine	106.6 ± 3.5	1.3	210.0 ± 10.7
SCH23390	>10000	-	>10000
L-741626	821.0 ± 103.0	9.7	274.0 ± 38.0
S-33084	59.0 ± 1.0	0.7	1.5 ± 0.2
BP-897	19.6 ± 1.9	0.2	0.4 ± 0.05
L-745870	>10000	-	>10000

Agonist activity was assayed by concentration response curves of various compounds with different reported selectivities for D_3 and percent efficacy normalized to 10 μM dopamine. IC_{50}s determined in the presence of 1 μM dopamine. K_bs were calculated by the method of Cheng–Prusoff [21]. These studies were complemented with binding using the D_2-like agonist radioligand [^{3}H]-DPAT. All determinations represent the mean ± S.E.M., $n = 4$.

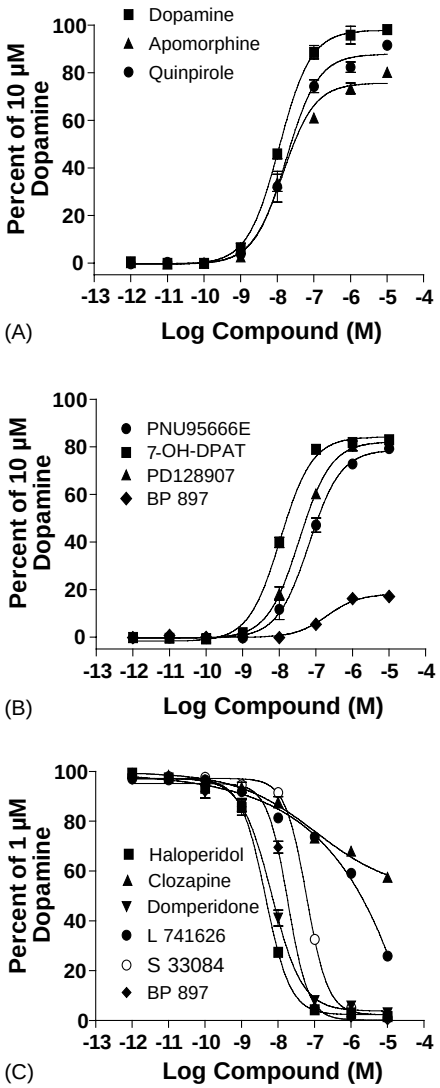

Fig. 5. Effects of dopamine agonists and antagonists on D_3-HEK cell line. Panel (A): effects of D_2-like agonists dopamine (closed squares), apomorphine (closed triangles) and quinpirole (closed circles). Panel (B): effects of D_2 agonists PNU95666E (closed circles), D_3 agonists 7-OH-DPAT (closed squares), PD128907 (closed triangles) and BP897 (closed diamonds). Panel (C): effects of antagonists against 1 μM dopamine. The cells were pre-incubated with various concentrations of haloperidol (closed squares), clozapine (closed triangles), domperidone (closed inverted triangles), L-741626 (closed circles), S33084 (open circles) and BP897 (closed diamonds) for 5 min at room temperature, add 1 μM of dopamine to measure the calcium response by the FLIPR. Data points represent mean ± S.E.M. $n = 4$.

D_1-selective agonist SKF81297 and D_4-selective agonists PD168077 and CP226269 showed very weak activities at D_3, confirming the selectivity of the assay (Table 4). However, the reported D_2 selective agonist PNU-95666E showed significant activity ($EC_{50} = 62.2$ nM with 79% efficacy of dopamine) at D_3 as well as activity in the D_3 binding assay ($K_i = 412$ nM) indicating that PNU-95666E cross-reacts with the D_3 receptor (Table 3). Reported D_3 agonists 7-OH-DPAT, PD128907 and BP897 were also tested in the $D_3/G\alpha_{qo5}$ cell line. 7-hydroxy-DPAT, and PD128907 showed D_3 agonist activities with $EC_{50} = 13.5$ and 29.7 nM with 81 and 82% efficacy, respectively (Table 4). Reported partial agonist BP897 showed only 16% efficacy of 10 μM dopamine (Fig. 5). D_4-selective agonist PD168077 had no detectable D_3 agonist activity up to 10 μM and weak binding on D_3 (~2.5 μM) while CP226269 exhibited weak agonist activity (~1 μM) as well as weak binding (0.5 μM, Table 4).

Dopamine receptor antagonists activities were determined in the presence of 1 μM dopamine in the $D_3/G\alpha_{qo5}$ cell line (Table 4). All the antagonists tested did not show any calcium signal by themselves (data not shown) indicating that the compounds have no intrinsic D_3 agonist activities. D_2-like antagonists, haloperidol and domperidone showed concentration-dependent inhibition of dopamine response (Fig. 5, Table 4). D_3 selective antagonist S33084 potently inhibited dopamine response ($K_b = 0.7$ nM), whereas D_1-selective antagonist SCH23390 and D_4 antagonist L-745870 did not inhibit the dopamine response (Table 4). The reported D_2 selective antagonist L-741626 also had potent D_3 activity ($K_b = 9.7$ nM) in this assay. BP897, in addition to showing partial D_3 agonist activity, is a potent D_3 antagonist ($K_b = 0.2$ nM).

4. Discussion

The preparation of stable HEK-293 cell lines transfected with recombinant human D_{2L}, D_3 or D_4 receptors and the chimeric G-protein $G\alpha_{qo5}$ cDNA has allowed a rapid and sensitive means to measure receptor-activated increases in intracellular calcium levels and compare dopaminergic agents in the same cellular background to assess receptor activation or inhibition and selectivity. In this report, we demonstrate that the human D_4 receptor can be coupled to calcium using a chimeric G-protein $G\alpha_{qo5}$ and that both agonists and antagonists can be detected in an assay that is also amenable to high throughput testing [12,27]. It was also shown that human D_{2L} and D_3 cell lines could be prepared and used for head to head comparisons of the three receptors in the same cellular background.

Dopamine D_2-like receptors naturally signal via $G\alpha_{i/o}$ [2,28]. In these assay systems, the chimeric G-protein, $G\alpha_{qo5}$, has been employed to facilitate $G_{i/o}$-coupled D_2-like receptors to mobilize intracellular calcium stores in response to agonist stimulation. Many $G_{i/o}$-coupled receptors such as opioid, chemokine and somatostatin receptors efficiently activate chimeric G-proteins, including $G\alpha_{16}$, $G\alpha_{qo5}$ and $G\alpha_{qi5}$, and stimulate calcium flux, but depending on the receptors and chimeric G-proteins, these recombinant systems do not always activate the chimeric G-proteins equally [12,29]. In the case of D_2-like receptors reported here, only $G\alpha_{qo5}$ yielded robust, transient calcium signal. Using Gα protein gene knock-out-mice, it has been demonstrated that dopamine D_2-like receptors in the brain couple primarily through $G\alpha_o$ in vivo [28]. This same G-protein preference has also been demonstrated in vitro for

D_{2L} [30,31] and for D_4 [32] using cloned receptors and transiently transfected cells. All of these reports as well as the data presented here are consistent with D_2-like receptors signaling through $G\alpha_o$.

The pharmacological characterization of dopaminergic agents across the three cell lines in our lab has revealed new information. The reported D_2-selective agonist PNU95666E [24] is equally potent in this report at human D_{2L} receptors as D_3, which is in agreement with agonist radioligand binding (Tables 3 and 4). This compound is devoid of any activity at the human $D_{4.4}$ receptor. The D_3 agonist activity in these assays suggests that in vivo effects of this compound may not be purely due to the D_2 receptor. Likewise, the reported D_3 agonists 7-hydroxy-DPAT and PD128907 are potent, full agonists across all three D_2-like receptors. The activation of D_2 and D_3 is consistent with both 7-hydroxy-DPAT and PD128907 showing decreases in body temperature and locomotor activity in D_3-knock-out mice that were not observed in D_2-knock-out mice, suggesting D_2 agonist activity [33,34]. Further, conclusions made from in vivo studies based on in vitro binding data using [^{3}H]-spiperone can lead to apparently conflicting data [18,35]. In this study, we confirm the weak partial agonist activity reported for BP-897 [25] but also demonstrate that this compound is a potent D_3 antagonist ($K_b = 0.2$ nM), consistent with recent reports [36,37]. In addition, this compound is also a 66% D_4 agonist ($EC_{50} = 485.5$ nM). In examining antagonists, clozapine is only 1.6-fold selective in this assay for D_4 over D_2. Our results confirm those in the literature as to the selectivity of S33084 and L-745870 for D_3 and D_4 respectively but fail to demonstrate D_2 selectivity for L-741626. This may explain in part the profile of inhibition of 7-hydroxy-DPAT and PD128907 in rat CNS studies by L-741626 [35] and emphasizes the need to assess functional selectivity as well as binding affinity before employing dopaminergic tools in vivo. The selective D_4 antagonist L-745870 [38] has been reported to as a partial agonist in cAMP assays ($EC_{50} = 2.5$ nM and 45% of dopamine) [39]. Using calcium flux assays, L-745870 was shown to exhibit partial agonist activity only at high concentrations but was a potent antagonist when assayed in the presence of dopamine. This reflects the dual nature of the pharmacology of this class of compounds [40].

Selectivity for D_2-like ligands has traditionally been defined by comparing binding affinities using membranes from cells that express recombinant receptors [2,41]. This approach can lead to a high degree of variability depending on the cell line used, assay conditions or radioligand employed. D_2-like receptors show discrimination for agonist and antagonist ligands depending on the nature of the competing radioligand. For example, D_2 binding affinities can differ up to 1000-fold for reported agonists depending on the radioligand [18] while D_4 shows similar phenomenon, but with four and five-fold differences [20]. For these reasons, we utilized agonist radioligands (D_2, PIPAT; D_3, 7-hydroxy-DPAT; D_4, A-369508) to determine binding affinities for both agonists and antagonists at all three receptors. In general, there the EC_{50}s determined from calcium flux assays and K_i from competitive binding are comparable. However, while receptor binding can determine the avidity of a compound for the receptor, it does not give any information regarding functional activity at these receptors (agonist or antagonist and relative potencies). Consequently, we have developed a novel functional assay to evaluate agonist and antagonist ligands of these receptors in a head to head fashion.

The concept of agonist trafficking has been introduced to discuss explain the phenomenon that GPCRs coupling to different G-proteins can give different agonist/antagonist potencies depending on the G-protein [42]. The finding that the five amino acid carboxy-terminal domain distinguishes $G\alpha_i$ from $G\alpha_o$ and $G\alpha_q$ facilitates $G\alpha_q$ mobilization

Table 5
Comparison of FLIPR and cAMP potencies and efficacies for human $D_{4.4}$ and D_{2L} receptor cell lines

Compound	Human D_{2L} FLIPR		Human D_{2L} cAMP	
	EC_{50} (nM)	Efficacy (%)	EC_{50} (nM)	Efficacy (%)
Dopamine	18.0 ± 2.0	100	25.9 ± 1.2	100
Apomorphine	5.8 ± 0.3	86	3.1 ± 1.3	95
Quinpirole	40.0 ± 4.0	90	14.3 ± 1.4	99
7-OH-DPAT	6.1 ± 0.6	84	9.6 ± 1.1	90
PD128907	22.0 ± 3.0	95	28.0 ± 1.4	102
	Human $D_{4.4}$ FLIPR		Human $D_{4.4}$ cAMP	
	EC_{50} (nM)	Efficacy (%)	EC_{50} (nM)	Efficacy (%)
Dopamine	2.2 ± 0.2	100	10.7 ± 1.1	100
Apomorphine	4.3 ± 0.2	84	0.8 ± 0.1	70
Quinpirole	20.6 ± 1.3	94	8.7 ± 2.1	90
PD168077	5.6 ± 0.7	68	3.0 ± 1.1	62

FLIPR and cAMP for human $D_{4.4}$ and FLIPR for human D_{2L} carried out as described in the methods. Human D_{2L} cAMP data from [44].

of calcium flux by $G\alpha_{i/o}$-coupled receptors [12,13]. It should be noted that despite the fact that this is not the physiological G-protein, this method does allow a means of coupling $G\alpha_{i/o}$ linked receptors to calcium flux using the five amino acid recognition sequence. One caveat is that other regions of the G-protein may be important for interaction with the receptor and can potentially influence signal transduction [43]. While the system described here is a sensitive means to detect agonist activity, what evidence is there to confirm measured efficacies and potencies correlate to the naturally coupled system? Inhibition of forskolin-induced cAMP in human $D_{4.4}$ transfected CHO cells is shown in Table 4 compared to the data generated in this report using chimeric G-proteins and calcium flux assays. All of the EC_{50}s of all compounds examined ranged from 0.8 to 21 nM and the percent efficacies in the two assays are comparable except for apomorphine, which is less than 15% efficacious in cAMP versus calcium flux. Also cAMP data from the literature on apomorphine, quinpirole, 7-hydroxy-DPAT and PD128907 for human D_{2L} [44] show the same rank order potencies and efficacies as the FLIPR assay (Table 5). While agonist trafficking could account for some of the differences between methods, the assays described in this report can distinguish agonists from antagonists and allow comparison across the three D_2-like receptors in the same cellular background.

The activation of D_4 receptors has been proposed as an important pathway in the central nervous system control of penile erection [7,8] as well as potentially important in cognition [4]. The difficulties in ascertaining agonist activity and receptor selectivity are reflected in the variability of data and lack of comparison in a head to head fashion in the same cell background with similar G-protein coupling [2,11,41]. While D_2-like receptors are not normally coupled to $G\alpha_q$, the cell lines described in this report compare favorably with $G\alpha_{i/o}$ coupling (Table 5) and should facilitate the identification of molecules that may have therapeutic applications or be used as tools to explore the role of the dopamine D_4 receptor in the central nervous system.

In conclusion, the stable co-expression of D_2-like receptors with chimeric $G\alpha_{qo5}$ proteins in HEK-293 cells is an efficient approach to study receptor activation and to determine functional selectivity of available D_2-like agonists across human D_{2L}, D_3 and D_4 receptors and facilitates discovery of both agonists and antagonists at these receptors.

Acknowledgments

The authors thank Drs. Michael Jarvis and Andrew Stewart for a critical reading of the manuscript and Drs. Marlon Cowart, Mark Matulenko and Teodozji Kolasa for synthesis of reference compounds.

References

[1] Kebabian JW, Calne DB. Multiple receptors for dopamine. Nature 1979;277:93–6.
[2] Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine receptors: from structure to function. Physiol Rev 1998;78:189–225.
[3] Wong AH, Buckle CE, Van Tol HH. Polymorphisms in dopamine receptors: what do they tell us? Eur J Pharmacol 2000;410:183–203.
[4] Hrib NJ. The dopamine D_4 receptor: a controversial therapeutic target. Drugs of the Future 2000;25:587–611.
[5] Khan ZU, Gutierrez A, Martin R, Penafiel A, Rivera A, De La Calle A. Differential regional and cellular distribution of dopamine D_2-like receptors: an immunocytochemical study of subtype-specific antibodies in rat and human brain. J Comp Neurol 1998;402:353–71.
[6] Ariano MA, Wang J, Noblett KL, Larson ER, Sibley DR. Cellular distribution of the rat D_4 dopamine receptor protein in the CNS using anti-receptor antisera. Brain Res 1997;752:26–34.
[7] Hsieh GC, Hollingsworth PR, Martino B, Chang R, Terranova MA, O'Neill AB, et al. Central mechanisms regulating penile erection in conscious rats: the dopaminergic systems related to the pro-erectile effect of apomorphine. J Pharmacol Exp Ther 2004;308:330–8.
[8] Brioni JD, Moreland RB, Cowart M, Hsieh GC, Stewart AO, Hedlund P, et al. Activation of dopamine D_4 receptors by ABT-724 facilitates penile erection in rats. Proc Natl Acad Sci USA 2004;101:6758–63.
[9] Asghari V, Sanyal S, Buchwaldt S, Paterson A, Jovanovic V, Van Tol HH. Modulation of intracellular cyclic AMP levels by different human dopamine D_4 receptor variants. J Neurochem 1997;65:1157–65.
[10] Newman-Tancredi A, Audinot V, Chaput C, Verriele L, Millan MJ. [^{35}S] Guanosine-5′-O-(3-thio) triphosphate binding as a measure of efficacy at human recombinant dopamine $D_{4.4}$ receptors: action of anti-Parkinsonian and antipsychotic agents. J Pharmacol Exp Ther 1997;282:181–91.
[11] Millan MJ, Maiofiss L, Cussac D, Audinot V, Boutin JA, Newman-Tancredi A. Differential actions of antiparkinson agents at multiple classes of monoaminergic receptor. I. A multivariate analysis of the binding profiles of 14 drugs at 21 native and cloned human receptor subtypes. J Pharmacol Exp Ther 2002;303:791–804.
[12] Coward P, Chan SD, Wada HG, Humphries GM, Conklin BR. Chimeric G proteins allow a high-throughput signaling assay of Gi-coupled receptors. Anal Biochem 1999;270:242–8.
[13] Coward P, Wada HG, Falk MS, Chan SD, Meng F, Akil H, et al. Controlling signaling with a specifically designed G_i-coupled receptor. Proc Natl Acad Soc USA 1998;95:352–7.
[14] Van Tol HH, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, et al. Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. Nature 1991;350:610–4.
[15] Rao VB. Direct sequencing of polymerase chain reaction-amplified DNA. Anal Biochem 1994;216:1–14.
[16] Gazi L, Wurch T, Lopez-Gimenez JF, Pauwels PJ, Strange PG. Pharmacological analysis of a dopamine D_{2short}:$G\alpha_o$ fusion protein expressed in Sf9 cells. FEBS Lett 2003;545:155–60.
[17] Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC. Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. Nature 1990;347:146–51.
[18] Chang RC, Hsieh GC, Brioni JD, Moreland RB. Differential competition of [^{125}I]-PIPAT and [^{3}H]-spiperone with dopamine agonist ligands at human D_2. Abstract Viewer/Itinerary Planner. Online Program No. 830.9. Society for Neuroscience:Washington, DC;2002.
[19] Vessotskie JM, Kung MP, Chumpradit S, Kung HF. Characterization of [^{125}I]S(−)5-OH-PIPAT binding to dopamine D_2-like receptors expressed in cell lines. Neuropharmacology 1997;36:999–1007.
[20] Moreland RB, Terranova MA, Chang R, Uchic ME, Matulenko MA, Surber BW, Stewart AO, Brioni JD. [^{3}H]-A-369508 ([2-[4-(2-cyanophenyl)-1-piperazinyl]-*N*-(3-methylphenyl) acetamide): an agonist radioligand selective for the dopamine D_4 receptor, Eur J Pharmacol, in press.

[21] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 1973;22:3099–108.
[22] Zorn SH, Jackson E, Johnson C, Lewis J, Fliri A. CP-226269 is a selective dopamine D_4 agonist. Soc Neurosci Abstr 1997;23:685.
[23] Glase SA, Akunne HC, Georgic LM, Heffner TG, MacKenzie RG, Manley PJ, et al. Substituted [(4-phenylpiperazinyl)-methyl]benzamides: selective dopamine D_4 agonists. J Med Chem 1997;40:1771–2.
[24] Heier RF, Dolak LA, Duncan JN, Hyslop DK, Lipton MF, Martin IJ, et al. Synthesis and biological activities of (*R*)-5,6-dihydro-*N*,*N*-dimethyl-4H-imidazo[4,5,1-ij]quinolin-5-amine and its metabolites. J Med Chem 1997;40:639–46.
[25] Pilla M, Perachon S, Sautel F, Garrido F, Mann A, Wermuth CG, et al. Selective inhibition of cocaine-seeking behaviour by a partial dopamine D_3 receptor agonist. Nature 1999;400(6742):371–5.
[26] Millan MJ, Gobert A, Newman-Tancredi A, Lejeune F, Cussac D, Rivet JM, et al. S33084, a novel, potent, selective, and competitive antagonist at dopamine D_3-receptors: I. receptorial, electrophysiological and neurochemical profile compared with GR218,231 and L741,626. J Pharmacol Exp Ther 2000;293:1048–62.
[27] Gopalakrishnan SM, Moreland RB, Kofron JL, Helfrich RJ, Gubbins E, McGowen J, et al. A cell-based micro-arrayed compound screening format for identifying modulators of G-protein coupled receptors: application for pharmacological studies and high throughput screening. Anal Biochem 2003;321:192–201.
[28] Jiang M, Spicher K, Boulay G, Wang Y, Birnbaumer L. Most central nervous system D_2 dopamine receptors are coupled to their effectors by G_o. Proc Natl Acad Sci USA 2001;98:3577–82.
[29] Kostenis E. Is $G\alpha_{16}$ the optimal tool for fishing ligands of orphan G-protein-coupled receptors? Trends Pharmacol Sci 2001;22:560–4.
[30] Pauwels PJ, Tardif S, Colpaert FC. Differential signaling of both wild-type and Thr^{343}Arg dopamine D_{2short} receptor by partial agonists in a G-protein-dependent manner. Biochem Pharmacol 2001;62:723–32.
[31] Courdeaux Y, Nickolls SA, Flood LA, Graber SG, Strange PG. Agonist regulation of D_2 dopamine receptor/G protein interaction. Evidence for agonist selection of G protein subtype. J Biol Chem 2001;276:28667–75.
[32] Kazmi MA, Snyder LA, Cypess AM, Graber SG, Sakmar TP. Selective reconstitution of human D_4 dopamine receptor variants with G_i alpha subtypes. Biochemistry 2000;39:3734–44.
[33] Boulay D, Depoortere R, Perrault G, Borrelli E, Sanger DJ. Dopamine D_2 receptor knock-out mice are insensitive to the hypolocomotor and hypothermic effects of dopamine D_2/D_3 receptor agonists. Neuropharmacology 1999;38:1389–996.
[34] Boulay D, Depoortere R, Rostene W, Perrault G, Sanger DJ. Dopamine D_3 receptor agonists produce similar decreases in body temperature and locomotor activity in D_3 knock-out and wild-type mice. Neuropharmacology 1999;38:555–65.
[35] Bristow LJ, Cook GP, Patel S, Curtis N, Mawer I, Kulagowski JJ. Discriminative stimulus properties of the putative dopamine D_3 receptor agonist, (+)-PD 128907: role of presynaptic dopamine D_2 autoreceptors. Neuropharmacology 1998;37:793–802.
[36] Wicke K, Garcia-Ladona J. The dopamine D_3 receptor partial agonist, BP 897, is an antagonist at human dopamine D_3 receptors and at rat somatodendritic dopamine D_3 receptors. Eur J Pharmacol 2001; 424:85–90.
[37] Wood MD, Boyfield I, Nash DJ, Jewitt FR, Avenell KY, Riley GJ. Evidence for antagonist activity of the dopamine D_3 receptor partial agonist, BP 897, at human dopamine D_3 receptor. Eur J Pharmacol 2000;407:47–51.
[38] Patel S, Freedman S, Chapman KL, Emms F, Fletcher AE, Knowles M, et al. Biological profile of L-745,870, a selective antagonist with high affinity for the dopamine D_4 receptor. J Pharmacol Exp Ther 1997;283:636–47.
[39] Gazi L, Bobirnac I, Danzeisen M, Schupbach E, Langenegger D, Sommer B, et al. Receptor density as a factor governing the efficacy of the dopamine D_4 receptor ligands, L-745,870 and U-101958 at human recombinant $D_{4.4}$ receptors expressed in CHO cells. Br J Pharmacol 1999;128:613–20.
[40] Stewart AO, Cowart MD, Moreland RB, Latshaw SP, Matulenko MA, Bhatia PA, et al. Dopamine D_4 ligands and models of receptor activation: 2-(4-Pyrimidin-2-ylpiperazin-1-ylmethyl)-1H-benzimidazole and related heteroarylmethylarylpiperzines exhibit a substituent effect responsible for additional efficacy tuning. J Med Chem 2004;47:2348–55.
[41] Vallone D, Picetti R, Borrelli E. Structure and function of dopamine receptors. Neurosci Biobehav Rev 2000;24:125–32.
[42] Kenakin T. Drug efficacy at G-protein-coupled receptors. Annu Rev Pharmacol Toxicol 2002;42:349–79.
[43] Slessareva JE, Ma H, Depree KM, Flood LA, Bae H, Cabrera-Vera TM, et al. Closely related G-protein-coupled receptors use multiple and distinct domains on G-protein alpha-subunits for selective coupling. J Biol Chem 2003;278:50530–6.
[44] Hall DA, Strange PG. Comparison of the ability of dopamine receptor agonists to inhibit forskolin-stimulated adenosine 3′ 5′-cyclic monophosphate (cAMP) accumulation via D_{2L} (long isoform) and D_3 receptors expressed in Chinese hamster ovary (CHO) cells. Biochem Pharmacol 1999;58:285–9.