Quantitative Comparison of Functional Screening by Measuring Intracellular Ca^{2+} With Radioligand Binding at Recombinant Human Dopamine Receptors

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ABSTRACT The purpose of this study was to test whether screening at dopamine receptors performed with a recently described functional assay for G-protein coupled receptors (GPCRs) provides data that correlate significantly with radioligand binding data in the literature, thus possibly allowing researchers to replace radioligand binding with nonradioactive functional screening. Human dopamine receptors hD1 and hD2L (representing G_s [hD1] or G [hD2L] coupled GPCRs) were recombinantly expressed in human embryonic kidney (HEK293) cells. Cells were loaded with Oregon Green 488 BAPTA-1/AM and evenly distributed in 384 well plates. Seventeen test compounds were screened for agonistic activity by injection into the cell suspension and monitoring of intracellular Ca^{2+} with a fluorescence microplate reader. Then, standard agonists (100nM SKF38393 for hD1, 30nM quinpirole for hD2L) were injected into wells preincubated with test compounds (screening for antagonism). Injection of various agonists resulted in a concentration-dependent increase in fluorescence. Further, preincubation of antagonists with dopamine receptor expressing cells inhibits concentration-dependent the agonist-induced increase in fluorescence. Calculated apparent functional K_i values correlate with radioligand binding data in the literature ($r^2 = 0.7796$ for D1, $r^2 = 0.7743$ for D2). The correlation between apparent functional K values and radioligand binding data for the 17 tested compounds suggests that screening of test compounds at dopamine receptors with the functional Ca^{2+} assay can replace radioligand binding studies. Furthermore, besides apparent K values, information about agonistic or antagonistic properties of a test compound can be obtained with the functional Ca^{2+} assay.

KEYWORDS: dopamine receptor, functional screening, Ca^{2+} assay, microplate reader, radioligand binding.

INTRODUCTION

G-protein coupled receptors (GPCRs) represent major therapeutic targets.¹ The human genome harbors

approximately 630 GPCRs.² These data underline the importance of high-throughput screening to find ligands at GPCRs as therapeutic leads both for well-known receptors and for orphan receptors. Dopamine receptors are involved in disorders like Parkinson's disease or schizophrenia and therefore represent important targets for these diseases.³ Two classes of human dopamine receptors can be distinguished: G_s coupled D1-like receptors (hD1 and hD5 receptors), and G coupled D2-like receptors (hD2, hD3, and hD4 receptors).⁴ Even though dopaminergic agonists and antagonists with a certain subtype selectivity are available,⁵ their therapeutic use is limited because of severe side effects — for instance, extrapyramidal motor effects of haloperidol and phenothiazines, or severe side effects of clozapine.⁶⁻⁸ The therapeutic long-term benefit and adverse drug reactions of new atypical neuroleptics like ziprasidone still need further evaluation.⁹ Therefore, the synthesis of novel ligands without therapy-limiting side effects, better subtype selectivity and specificity, and subsequent screening of compound libraries are still under thorough investigation in the dopaminergic field. So far, high-throughput radioligand binding methods as well as some nonradioactive screening systems (eg, fluorescence polarization) have been established.¹⁰ However, what is needed are simple and robust screening systems that can be performed in laboratories with "low to medium" throughput and avoid the use of radioisotopes. Further, these screening methods should allow a simple functional discrimination between agonists and antagonists and be ideally applicable to a majority of receptors. Therefore, a functional screening method for GPCRs was recently developed based on the increase in intracellular Ca^{2+} upon agonist stimulation measured with the Ca^{2+} binding fluorescent dye Oregon Green 488 BAPTA-1.¹¹ Ca^{2+} is a near universal signal for not only ion channels but also recombinantly expressed GPCRs.¹² The functional Ca^{2+} assay allows screening at various G_s , G , G_q , and G_o coupled receptors, among them the recombinantly expressed human dopamine receptors hD1 and hD2L.¹¹ Further, the assay can be performed in a 384 well plate format, thus allowing a medium-throughput screening. The Ca^{2+} assay distinguishes between agonists and antagonists, and yields information about the potency of an agonist (EC_{50} : effective concentration 50%) or an antagonist (IC_{50} : inhibitory concentration 50%, or apparent K value: apparent inhibition constant calculated according to the Cheng-Prusoff equation).¹³ For many well-known ligands, K values obtained by radioligand binding exist in the literature.⁵ However, to test whether K values

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obtained by radioligand binding are comparable and correlate with apparent K values obtained with the functional Ca^{2+} assay, a larger number of ligands—well characterized in terms of radioligand binding—need to be examined with the functional Ca^{2+} assay. Since dopamine receptors represent important therapeutic targets and various dopaminergic ligands (agonists and antagonists) are readily available, the aim of this study was to test whether apparent K values obtained from the novel functional Ca^{2+} assay are significantly correlated with K values obtained by radioligand binding studies. The study was performed with recombinantly expressed human dopamine receptors hD1 and hD2L — 1 representative from each of the 2 major classes of G_s and G coupled GPCRs. Seventeen known dopaminergic ligands, among them 7 agonists and 10 antagonists, were tested for agonistic or antagonistic behavior in the Ca^{2+} assay. Obtained EC₅₀ or apparent K values were correlated with literature-based K values previously measured by equilibrium radioligand binding.

MATERIALS AND METHODS

Cell Culture

Stable transfected human embryonic kidney (HEK293) clonal cell lines were cultured in Dulbecco's modified Eagle Medium Nutrient Mixture F-12 Ham (DMEM/F12 1:1 Mixture) (Sigma Chemical, Taufkirchen, Germany) containing 100 µg/mL streptomycin, 100 U/mL penicillin G, 10% fetal bovine serum (Sigma Chemical), 5mM L-glutamine (Sigma Chemical), and 200 µg/mL G-418 (Calbiochem-Novabiochem Corporation, San Diego, CA). Cells were incubated at 37°C in 5% CO₂.

Stable Transfection of HEK293 Cells

Stable cell lines of HEK293 cells (ATCC, Rockville, MD) were prepared by transfection of pcDNA3 containing a fragment coding for the entire human dopamine receptor hD1 or hD2L, respectively. Transfection was performed with the Roti-Fect transfection reagent (Roth, Karlsruhe, Germany). The pcDNA3-hD1 vector was obtained by subcloning of the hD1 cDNA from pGEM3-hD1 plasmid into the EcoRI and XbaI sites of pcDNA3 (Invitrogen Corporation, Carlsbad, CA). Clonal selection was performed by adding 400 µg/mL G-418 to the culture medium. The pcDNA3-hD2L vector was obtained from Dr W. Sadée (San Francisco, CA). Expression levels (B_{max}) and dissociation equilibrium constants (K_d) for standard ligands ($[^3\text{H}]\text{-SCH23390}$, $[^3\text{H}]\text{-spiperone}$) at hD1 or hD2L receptor expressing HEK293 cells are listed in Kassack et al.¹¹

Materials

cDNA for hD1 cloned in pGEM3 (Promega, Madison, WI) was obtained from Dr David Grandy (Portland, OR). Compound LE300 was synthesized in the group of Dr Jochen Lehmann (Bonn, Germany), according to Witt et al.¹⁴ All other compounds and reagents were obtained at Sigma Chemical unless otherwise stated.

Radioligand Binding of LE300 at hD1 and hD2L Dopamine Receptors in HEK293 Cells

K values of LE300 at hD1 and hD2L receptors were obtained as previously described.^{11,14} Briefly, 1 T75 flask of HEK293 cells (~90% confluent) recombinantly expressing the respective receptor was harvested by trypsinization (0.05% trypsin/0.02% EDTA), and cells were resuspended in phosphate-buffered saline (PBS), pelleted, and rinsed once with PBS. Pelleted cells were then resuspended in Krebs-HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffer (118mM NaCl, 4.7mM KCl, 1.2mM MgSO₄, 1.2mM KH₂PO₄, 4.2mM NaHCO₃, 11.7mM D-glucose, 1.3mM CaCl₂, 10mM HEPES, pH 7.4) and evenly distributed into pretreated microcentrifuge tubes (Sigma-cote). Binding assays were carried out in triplicate in a total volume of 1 mL. Incubation was initiated by addition of 100 µL of $[^3\text{H}]\text{-ligand}$ and was carried out at 27°C for 1 hour. It was stopped by rapid filtration through pretreated glass fiber filters (0.25% polyethyleneimine), followed by 2 x 5 mL washes with ice cold water. Radioactivity retained on the filters was counted by liquid scintillation spectroscopy using a Beckman scintillation counter. Radioligands were added at a nonsaturating concentration. For binding at hD1 receptors, 0.2nM [N-methyl- $[^3\text{H}]\text{-SCH23390}$ (83.0 Ci/mmol, Nycomed Amersham, Buckinghamshire, England) was used. Nonspecific binding was determined using SCH23390 (Sigma Chemical) in a final concentration of 2µM. Then, 0.1nM $[^3\text{H}]\text{-spiperone}$ (97.0 Ci/mmol, Nycomed Amersham) was used for binding studies at the hD2L receptor. Nonspecific binding was determined using spiperone in a final concentration of 1µM. Total protein was estimated using the Bradford method.¹⁵ LE300 was added in increasing concentrations.

Radioligand binding data for standard ligands at dopamine receptors were obtained from the literature or the Web site, as stated in **Tables 1** and **2**.

Measurement of Intracellular Ca²⁺ in HEK293 Cells

Ca²⁺ fluorescence measurements were performed using a NOVOstar microplate reader (BMG LabTechnologies, Offenburg, Germany) equipped with a pipettor and 2 injectors as previously described.¹¹ HEK293 cells expressing the respective dopamine receptor were harvested with 0.05% trypsin/0.02% EDTA and rinsed with culture medium containing 10% fetal bovine serum, 100 µg/mL streptomycin, 100 U/mL penicillin G, and 1mM L-glutamine. Pelleted cells were then resuspended in fresh medium. They were kept under 5% CO₂ at 37°C for 45 minutes and vortexed every 15 minutes. After 2 washes with Krebs-HEPES buffer, cells were loaded with 3µM Oregon Green 488 BAPTA-1/AM (Molecular Probes, Eugene, OR) for 1 hour at 25°C in the same buffer containing 1% Pluronic F-127. Then, cells were rinsed 3 times with Krebs-HEPES buffer containing 0.5% bovine serum albumin, counted by using a Casy I Model TT cell

counter device (Schaerfe System GmbH, Reutlingen, Germany); diluted; and evenly plated into black 384 well plates with clear bottom (Greiner, Frickenhausen, Germany) at a density of approximately 15 000 cells/well. The volume of cell suspension added to each well was 64 μ L. Microplates were kept at 37°C. Fluorescence intensity was measured at 520 nm (bandwidth 35 nm) for 7 seconds at 1.0-second intervals to monitor baseline. After 8 seconds, 8 μ L of buffer alone or test compounds (agonists or antagonists) dissolved in buffer were then injected sequentially into separate wells by using the pipettor, and fluorescence intensity was monitored at 520 nm (bandwidth 35 nm) for 22 seconds at 0.4-second intervals. Excitation wavelength was 485 nm (bandwidth 12 nm). After approximately 45 minutes of incubation, 8 μ L of a standard agonist in a concentration of $\sim 5 \times EC_{50}$ (100 nM SKF38393 for hD1, and 30 nM quinpirole for hD2L receptors) was injected into the same wells (containing cells and test compounds [agonists or antagonists]), thus allowing estimation of the ability of a test compound to block agonist-induced increase in intracellular Ca^{2+} and to calculate an apparent K value. Intracellular Ca^{2+} concentration ($[Ca^{2+}]$) was calculated as follows³⁵:

$$[Ca^{2+}] = K_d (F - F_{min}) / (F_{max} - F).$$

F_{max} refers to fluorescence intensity measured after permeabilization of the cells with 1% Triton X-100. Then, 10mM EDTA was added to chelate Ca^{2+} , and minimum fluorescence intensity was obtained (F_{min}). An equilibrium dissociation constant (K_d) of 170nM was used for Oregon Green 488 BAPTA-1 (Molecular Probes, Eugene, OR). Intracellular Ca^{2+} was estimated for each experiment to control for even loading of fluorescent dye. Quantitative data were obtained by estimating the maximum fluorescence intensity of each data set.

Data Analysis

Concentration response curves were constructed using nonlinear regression curve fit (sigmoidal dose response equation with variable hill slope), which was performed by using Prism software 3.0 from GraphPad. Hill slopes (n_H) were not significantly different from unity. Apparent K values for functional experiments (Ca^{2+} assay) were calculated according to the following equation adapted from Cheng and Prusoff¹³:

$$K_i = IC_{50} / (1 + L/EC_{50}) \quad (1)$$

where IC_{50} is the inhibitory concentration 50% of the antagonist, EC_{50} is the effective concentration 50% of the used agonist (ie, SKF38393 for hD1, and quinpirole for hD2L receptors), and L is the molar concentration of the used agonist.

The K value of LE300 obtained by radioligand binding experiments was calculated according to the equation of Cheng and Prusoff¹³:

$$K_i = IC_{50} / (1 + L/K_d) \quad (2)$$

where IC_{50} is the inhibitory concentration 50% of the antagonist LE300 (replacing 50% of bound radioactivity), K_d is the dissociation equilibrium constant of the used radioligand, and L is the molar concentration of the used radioligand.

RESULTS

The recently developed functional Ca^{2+} assay served as a method of screening at human dopamine receptors.¹¹ A reproducible concentration-dependent Ca^{2+} signal, measured as increase in fluorescence upon Ca^{2+} binding to Oregon Green 488 BAPTA-1, was observed for agonist stimulation of hD1 and hD2L receptors, respectively (Figure 1, Figure 2). SKF38393 serves as an example of an agonist at hD1 receptors in Figure 1, pergolide as agonist for hD2L receptors in Figure 2. Injection of increasing concentrations of known antagonists of hD1 or hD2L receptors did not change the basal fluorescence levels in either cell line, thus indicating that the observed increase in fluorescence after injection of increasing concentrations of agonist is mediated by receptors rather than mechanical stress (Figure 1, Figure 2). Further, injection of SKF38393 or pergolide into wildtype HEK293 cells lacking dopamine receptors did not yield any response up to 100 μ M (data not shown). One critical factor in yielding reproducible agonist concentration response curves turned out to be the method of harvesting the cells used for the Ca^{2+} assay. If cells were harvested using trypsin/EDTA and minimum application of mechanical forces (ie, no scraping of cells), dopamine receptor expressing HEK293 cells showed a large increase in fluorescence after injection of agonist, yielding a maximum response after approximately 15 seconds as shown for hD1 receptors stimulated with 100nM SKF38393 (Figure 3A, filled squares). However, if the same cells were harvested by mechanical scraping using cell scrapers, only a tiny response could be monitored after injection of 100nM SKF38393 (Figure 3A, open squares), peaking at approximately 3 seconds and completely disappearing within 10 seconds after injection of agonist. The results of the 2 different harvesting methods — ie, the cells — were therefore monitored after fluorescent dye loading with a Casy I Model TT cell counter device estimating the size distribution pattern of cells. As can be seen in representative examples in Figures 3B and 3C, the size distribution pattern changes dramatically if different cell harvesting methods are used. For both experiments, one T75 flask with 90% confluent hD1 expressing HEK293 cells was used, respectively; therefore, both experiments started with the same number of cells. However, harvesting cells by using trypsin/EDTA leaves the majority of cells with a mean diameter of 15 μ m and a total number of cells of 2.4×10^6 cells/mL in the range of 10 to 40 μ m (Figure 3B), whereas mechanical harvesting of cells with a cell scraper reduces the number of viable cells (10-40 μ m) dramatically and

Figure 1. Concentration-response curve of SKF38393 at the hD1 receptor recombinantly expressed in HEK293 cells. Response is measured as increase in fluorescence intensity upon increase in intracellular Ca^{2+} after stimulation of the hD1 receptor with the agonist SKF38393. Dopamine receptor antagonists (haloperidol, SCH23390, chlorprothixene) did not yield a response up to 10 μM (or 100 μM for SCH23390). Presented data are the average of 3 replicates $\pm$ SE and represent 3 independent experiments. EC_{50} of SKF38393 was calculated as 18.1 nM $\pm$ 8.8 (SD, $n = 3$).

Figure 2. Concentration-response curve of pergolide at the hD2L receptor recombinantly expressed in HEK293 cells. Response is measured as increase in fluorescence intensity upon increase in intracellular Ca^{2+} after stimulation of the hD2L receptor with the agonist pergolide. Dopamine receptor antagonists (clozapine, haloperidol, chlorprothixene) did not yield a response up to 10 μM . Presented data are the average of 3 replicates $\pm$ SE and represent 3 independent experiments. EC_{50} of pergolide was calculated as 4.1 nM $\pm$ 1.2 (SD, $n = 3$).

leaves a majority of shrunk cells (diameter $<10 \mu\text{m}$) or cell debris (**Figure 3C**). Thus, although the same number of cells were used at the start of both harvesting experiments, the outcomes are quite different: whereas harvesting with trypsin/EDTA leaves 2.4×10^6 viable cells/mL (diameter range of 10-40 μm), mechanical harvesting with a cell scraper yields only one third that number of viable cells (0.8×10^6 cells/mL in the range of 10-40 μm).

Seven agonists of dopamine receptors (hD1 and/or hD2) were tested for agonistic behavior in the Ca^{2+} assay by injection into a suspension of hD1 or hD2L receptor expressing cells, respectively, and Ca^{2+} mediated changes in fluorescence were monitored. Resulting EC_{50} values are listed in **Table 3**. All compounds known as potent

agonists at hD1 receptors also behaved as agonists in the Ca^{2+} assay. Bromocriptine, a compound with weak affinity at the hD1 receptor ($K = 440 \text{ nM}^5$); quinpirole, with very weak affinity at the hD1 receptor ($K = 1900 \text{ nM}^5$); and mesulergine did not display agonistic activity at the hD1 receptor ($\text{EC}_{50} > 10\,000 \text{ nM}$). Interestingly, even though pergolide, apomorphine, SKF38393, and lisuride are reported to have similar high-affinity binding at hD1 receptors ($K = 0.8, 0.7, 1.0$, and 5.6 nM for pergolide, apomorphine, SKF38393, and lisuride, respectively^{5,23}), their EC_{50} values differ (**Table 3**): apomorphine is ~4-fold, pergolide ~10-fold, and lisuride ~75-fold less potent than SKF38393 at hD1 receptors in the functional Ca^{2+} assay. Pergolide, apomorphine, and quinpirole are equipotent agonists in the low nanomolar range at hD2L receptors in the Ca^{2+} assay, as listed in **Table 3**. However, lisuride and

Figure 3. (A) Increase in fluorescence caused by an increase in intracellular Ca^{2+} due to stimulation of the hD1 receptor recombinantly expressed in HEK293 cells with 100 nM SKF38393. hD1 expressing cells were harvested with 0.05% trypsin/0.02% EDTA and minimized mechanical forces (no scraping) (■), or cells were harvested by mechanical scraping in the absence of trypsin/EDTA (□). Presented data are the average of 3 replicates $\pm$ SD and represent 3 independent experiments. (B, C) Size distribution of hD1 receptor expressing HEK293 cells harvested by (B) applying 0.05% trypsin/0.02% EDTA and minimized mechanical forces (no scraping), yielding curve ■ in Figure 3A; or by (C) mechanical scraping in the absence of trypsin/EDTA, yielding curve □ in Figure 3A. Number of cells in the range between 10 and 40 μm was estimated as 2.4×10^6 cells/mL (B) or 0.8×10^6 cells/mL (C). Mean diameter of the cells was calculated as 15.0 μm (B) or as 13.8 μm (C) in the range between 10 and 40 μm .

bromocriptine also display nanomolar affinity ($K = 0.8$ or 0.6nM for lisuride or bromocriptine, respectively^{29,31}) but are weak agonists in the Ca^{2+} assay at hD2L receptors ($\text{EC}_{50} = 175$ or 1635nM for lisuride or bromocriptine, respectively, **Table 3**). Mesulergine, described as a weak dopamine receptor agonist,^{31,36} displayed an EC_{50} of $\sim 10,000\text{nM}$ at hD2L receptors.

Next, 10 structurally different antagonists of hD1 and/or hD2 receptors were tested (**Table 4**). Upon injection of the antagonists into a hD1 or hD2L receptor expressing cell suspension, no change in baseline fluorescence occurred (eg, displayed for the antagonists haloperidol, SCH23390, and chlorprothixene at hD1 receptors, **Figure 1**, and displayed for clozapine, haloperidol, and chlorprothixene at hD2L receptors, **Figure 2**). After injection, antagonists were incubated with the receptor expressing cells for 45 minutes to allow equilibration. Then, in a second injection, a standard agonist (in a concentration of ~ 5 -fold EC_{50} , ie, 100nM SKF38393 for hD1 receptors, or 30nM quinpirole for hD2L receptors) was injected into the cell suspension preincubated with antagonists, and fluorescence was monitored. Thus, apparent K values for antagonists were obtained. However, the same procedure — injection of test compound, 45 minutes of equilibration, and subsequent injection of a standard agonist (SKF38393 or quinpirole for hD1 or hD2L receptors, respectively) — was applied for the agonist compounds in **Table 3**, therefore also yielding "apparent K values" as listed in the agonist portion of **Table 4**.

In the next step, apparent functional K values obtained by the Ca^{2+} assay were compared with literature-based K values obtained by radioligand binding (**Tables 1 and 2**, **Figures 4A and 4B**). If available, binding data were taken from human dopamine receptors. Otherwise, data were taken from rat, calf, or dog striatal receptors (**Tables 1 and 2**). Literature was searched to get an overview of the range of literature-based binding data, and lowest and highest found K values were listed (**Tables 1 and 2**). For agonists, K values for the high- and low-affinity binding sites are listed, respectively. For D1 receptors, literature-based K values are in a slightly narrower range (maximum 19.5-fold difference for SCH23390, **Table 1**) than literature-based K values for D2 receptors (27.4-fold difference for K values for haloperidol, 21.2-fold difference for chlorpromazine, 11-fold difference for clozapine, **Table 2**). Not surprisingly, there are big differences between high- and low-affinity binding sites for agonists at both D1 and D2 receptors (the agonist portions of **Tables 1 and 2**). LE300, a novel dopamine receptor antagonist, was included in this study because it contains a novel chemical structure for dopamine receptor antagonists (benz[d]indolo[2,3-g]azecine ring system) and has recently been described as a dopamine receptor antagonist.¹⁴ The purpose of this study was to test whether the functional Ca^{2+} assay can serve as a quantitative screening method to detect agonists and antagonists. Therefore, a correlation analysis of apparent functional with literature-based binding K values was performed. Results are presented in **Figures 4A and 4B**. Apparent functional K values of agonists were included and compared with the high-affinity binding K values from the literature in the correlation analysis (the agonist portions of **Tables 1 and 2**). Apparent functional K values of antagonists were compared with the lowest and highest binding K values from the literature (the antagonist portions of

Table 1. Comparison of Functional Versus Radioligand Binding Data for Various Dopamine Receptor Antagonists and Agonists at hD1 Receptors*

Compound	hD1: K _i (nM) Ca ²⁺ Assay	D1: K _i (nM) Binding	Binding K _i Literature
Antagonists			
SCH23390	0.8	0.2-3.9	16, 17
Chlorprothixene	48.3	4.0 [†]	18
LE300	60.4	1.9-4.9	
Fluphenazine	85.3	7.0-21.0	16, 19
Haloperidol	230	24.0-82.0	20, 21
Clozapine	250	141-196	16, 21
Levomepromazine	586	NA	
Spiperone	1080	220-577	16, 19
Chlorpromazine	1136	44.0-158	16, 22
Agonists			
Pergolide	0.8	0.8-1363 [‡]	5, 16
Lisuride	0.9	5.6 [§] -58 ^{‡§}	23
Apomorphine	1.2	0.7-680 [‡]	5, 16
SKF38393	2.8	1-987 [‡]	5, 19
Bromocriptine	460	440-1482	5, 24
Quinpirole	1532	1900	5

*Shown are apparent functional K_i values obtained by measuring the inhibition of agonist (100 nM SKF38393) induced increase in intracellular Ca²⁺ after preincubation with the respective test compound from the antagonist or the agonist group and subsequent injection of 100 nM SKF38393. Binding data are obtained from the cited literature, except for LE300, which was determined by radioligand binding, as previously described [11], and is given as the range between minimum and maximum K_i values found in the literature. NA indicates not available.

[†]Rat dopamine receptors.

[‡]High- or low-affinity status of receptors, respectively.

[§]Calf or dog striatal dopamine receptors.

Table 2. Comparison of Functional Versus Radioligand Binding Data for Various Dopamine Receptor Antagonists and Agonists at hD2L Receptors*

Compound	hD2: K _i (nM) Ca ²⁺ Assay	D2: K _i (nM) Binding	Binding K _i Literature
Antagonists			
Spiperone	0.2	0.06-0.13	25
Haloperidol	1.8	0.35-9.6	25, 26
Chlorprothixene	3.2	8.0	27
Levomepromazine	4.3	15 [†]	28
Fluphenazine	9.4	0.32-0.9	19, 26
Chlorpromazine	13.5	0.66-14	25, 26
LE300	19.0	44.7-83.4	
Clozapine	88.9	38-420	25, 29
SCH23390	2600	5560 [†]	30
Agonists			
Pergolide	0.3	0.8 [§] -137 [‡]	23, 31
Lisuride	0.6	0.8-4.8 [‡]	31, 32
Apomorphine	1.1	2.3-350 [‡]	32, 33
Bromocriptine	3.3	0.62-54 ^{‡§}	29, 30
Quinpirole	6.5	4.8-1402 [‡]	5, 34
SKF38393	~230	>10 000	19

*Shown are apparent functional K_i values obtained by measuring the inhibition of agonist (30 nM quinpirole) induced increase in intracellular Ca²⁺ after preincubation with the respective test compound from the antagonist or the agonist group and subsequent injection of 30 nM quinpirole. Binding data are obtained from the cited literature, except for LE300, which was determined by radioligand binding, as previously described [11], and is given as the range between minimum and maximum K_i values found in the literature.

[†]Rat dopamine receptors.

[‡]High- or low-affinity status of receptors, respectively.

[§]Calf or dog striatal dopamine receptors.

Tables 1 and 2). The overall correlation for D1 receptors yielded an r^2 of 0.7796, showing a significant correlation with $P < .0001$ (**Figure 4A**). The slope is not significantly different from 1 (95% confidence interval is between 0.72 and 1.24). For D2 receptors, the overall correlation gave an r^2 of 0.7743 (significant correlation with $P < .0001$ [**Figure 4B**]). In contrast to D1 receptors, the

slope for D2 receptor correlation is significantly different from 1 (95% confidence interval is between 0.51 and 0.85). These data suggest that apparent functional and radioligand binding K values are significantly correlated but that an increase in radioligand binding yields a smaller increase in functional effects (measured as Ca²⁺) in D2 receptor expressing cells.

Figure 4. Correlation of apparent functional pK_i values ($\pm$ SD, $n = 3$) with literature-based pK_i values obtained by radioligand binding at D1 (A) or D2 receptors (B), respectively. K_i of LE300 was estimated as previously described [11]. Apparent functional K_i values were obtained by measuring the inhibition of agonist (100nM SKF38393 at D1, 30nM quinpirole at D2 receptors) induced increase in intracellular Ca^{2+} after preincubation with the respective test compound. For agonists, only literature K_i values for the high-affinity receptor state were used. For antagonists, minimum and maximum K_i values found in the literature (see Tables 1 and 2) were used for correlation with apparent functional K_i values. r^2 was calculated as 0.7796 for D1 (A) and as 0.7743 for D2 receptors (B). For both receptors, a significant correlation ($P < .0001$) was obtained.

DISCUSSION

Screening of compounds at GPCRs is an important issue not only in high-throughput screening laboratories but also in situations where fewer compounds need to be tested. Besides the affinity of a compound, functional properties are important. Further, it is desirable for one assay to be able to be applied to as many GPCRs as possible. Recently, a functional assay based on the change in intracellular Ca^{2+} upon agonist stimulation was described for differently coupled GPCRs (G_s , G , G_q).¹¹ In this report, it was shown that compounds described in

the literature as agonists at D1 and/or D2 receptors are indeed capable of inducing a Ca^{2+} signal (Table 3). The specificity of this signal was proved by antagonist injection, which did not yield a Ca^{2+} response (Figure 1, Figure 2), and by injection of agonists into control cells lacking dopamine receptor expression (HEK293 wildtype cells, data not shown). Harvesting the dopamine receptor expressing HEK293 cells for the functional Ca^{2+} assay needs to be done carefully: cells must be harvested gently (by use of trypsin/EDTA and as little mechanical force as possible) to retain a maximum sensitivity (Figure 3A). The Ca^{2+} assay was performed with dopamine receptor expressing cells in suspension rather than attached cells because of the lack of a microplate washer. However, if a microplate washer is available, the Ca^{2+} assay may be performed with attached cells, too.¹¹ Known dopamine receptor antagonists from various chemical classes are capable of blocking the agonist response in a concentration-dependent manner in hD1 or hD2L receptor expressing cells, resulting in a list of apparent functional K values (Table 4). Thus, the Ca^{2+} assay reveals not only whether a test compound is a dopamine receptor ligand (apparent K value) but also whether it is an agonist or an antagonist.

The functional potency of agonists in the Ca^{2+} assay (ie, the EC_{50} values, Table 3) does not correlate with binding data in the literature (Tables 1 and 2). However, all literature-known hD1 or hD2 agonists showed an agonistic response in the Ca^{2+} assay (Table 3). There may be various reasons for differences between functional EC_{50} values and radioligand binding data (K values), as seen, for instance, for pergolide at the D1 receptor (high-affinity radioligand binding $K = 0.80$ nM,⁵ low-affinity radioligand binding $K = 1363$ nM,¹⁶ functional EC_{50} in the Ca^{2+} assay: 171nM, Table 3). First, dopamine receptors are known to exist in 2 different states: a high- and a low-affinity state for agonists.^{5,37} Especially in recombinant cell lines, the amount of available G-proteins for receptor coupling is limited, thus leading to a relevant number of uncoupled receptors in a low-affinity state.⁵ Further, different agonists may stabilize different conformations of the receptor with different abilities to couple to and activate G-proteins.³⁸ Second, Ca^{2+} is a very fast signal peaking after ~15 seconds, thus not allowing equilibration of the agonist with the receptor. This may lead to an incomplete monitoring of an agonist's potency when applying the functional Ca^{2+} assay. Third, since Ca^{2+} is a "third" messenger for G_s and G coupled receptors like hD1 and hD2L, a far downstream — but nevertheless universally applicable — signal is monitored. Different agonists may activate the Ca^{2+} pathway with different potency by inducing different receptor conformations.³⁸ Taken together, agonists may display different behavior in equilibrium radioligand displacement studies and in functional experiments like the Ca^{2+} assay. However, all potent literature-known agonists have been successfully identified with the Ca^{2+} assay as agonists (Table 3).

Table 3. EC₅₀ Values and SD (nM) of Known Dopamine Receptor Agonists at hD1 and hD2L Receptor Expressing HEK293 Cells*

Agonist	hD1		hD2L	
	EC ₅₀ , nM	SD	EC ₅₀ , nM	SD
Apomorphine	80.3	30.8	4.7	2.3
Bromocriptine	>10 000	ND	1635	163
Lisuride	1350	1104	175	37.8
Mesulergine	>10 000	ND	~10 000	ND
Pergolide	171	61.8	4.1	1.2
Quinpirole	>10 000	ND	6.5	2.3
SKF38393	18.1	8.8	1380	ND

*Data were obtained by measuring agonist-induced increase in intracellular Ca²⁺. EC₅₀ values and SD were calculated from 3 independent experiments. ND indicates not determined (compound considered inactive).

Table 4. Apparent K_i Values and SD (nM) of Dopamine Receptor Antagonists and Agonists at hD1 and hD2L Receptor Expressing HEK293 Cells*

Compound	hD1		hD2L	
	K _i , nM	SD	K _i , nM	SD
Antagonists				
Amantadine	>1500	NA	53.5	10.2
Chlorpromazine	1136	NA	13.5	4.0
Chlorprothixene	48.3	26.5	3.2	1.3
Clozapine	250	56.5	88.9	35.0
Fluphenazine	85.3	35.8	9.4	3.1
Haloperidol	230	64.1	1.8	1.4
LE300	60.4	20.4	19.0	11.7
Levomopromazine	586	191	4.3	1.3
SCH23390	0.8	0.3	2600	850
Spiperone	1080	81.0	0.16	0.01
Agonists				
Apomorphine	1.2	0.4	1.1	0.6
Bromocriptine	460	124	3.3	0.8
Lisuride	0.9	0.2	0.6	0.4
Mesulergine	1629	503	28.6	15.9
Pergolide	0.8	0.3	0.3	0.2
Quinpirole	1532	NA	6.5	2.3
SKF38393	2.8	1.3	~230	NA

*Data were obtained by measuring the inhibition of agonist-induced increase in intracellular Ca²⁺. Test compounds were incubated for 45 minutes with hD1 or hD2L expressing HEK293 cells. Then, a standard agonist (100nM SKF38393 for hD1, and 30nM quinpirole for hD2L) was injected into a hD1 or hD2L expressing HEK293 cell suspension, and fluorescence intensity was monitored for 30 seconds. Concentration response curves were constructed by applying a sigmoidal concentration response curve fit. Apparent K_i values were estimated according to the equation from Cheng and Prusoff [13]. Apparent K_i values and SD were calculated from 3 independent experiments. If SD is not available, only 2 experiments were performed. NA indicates not available.

For the purpose of quantifying the effects of agonists by functional screening, the estimation of apparent functional K values (performed as described under "Materials and Methods") is better suited, as can be seen by comparison of apparent functional K values with high-affinity binding data (the agonist portions of **Tables 1** and **2**, "K (nM) Ca²⁺ Assay" compared with "K (nM) Binding"). Apparent functional K values of agonists are a measure for the dose-dependent functional blockage of dopamine receptors after continuous (45 minutes) preincubation with such agonistic test compounds. During the 45-minute preincubation with agonistic test compounds, dopamine receptors are first activated (which was not measured), and then as a consequence of continuous stimulation, as literature data suggest, desensitized and/or internalized.³⁹⁻⁴¹ After 45-minute incubation with agonistic test compounds, 100 nM SKF38393 for D1 or 30nM quinpirole for D2 receptors was injected. All agonistic test compounds were able to functionally block D1 or D2 receptors in a dose-dependent manner (the agonist portions of **Tables 1** and **2**). Further, apparent K values cal-

culated from the Ca²⁺ assay correlate well with literature-based high-affinity binding data (the agonist portions of **Tables 1** and **2**).

Mesulergine, a weak dopamine receptor agonist,^{36,42} was inactive at hD1 receptors and a very weak agonist at hD2L receptors in the Ca²⁺ assay (EC₅₀ ~10 000 nM). However, no radioligand binding data for mesulergine at dopamine receptors were found in the literature. Mesulergine is a potent antagonist at various serotonin receptors, especially the 5-HT_{2C} receptor.⁴³ Amantadine is used as a drug in the treatment of Parkinson's disease even though its efficacy is poor compared to L-dopa's.⁴⁴ Although the exact mechanism of action of amantadine against Parkinson's disease is still unknown, amantadine increases the dopaminergic tone without directly stimulating dopamine receptors.⁴⁵ In the Ca²⁺ assay, amantadine did not show agonistic activity at dopamine receptors, but, interestingly, amantadine inhibits the hD2L receptor with a K of 53.5nM (**Table 4**). These data, suggesting that amantadine is a moderate D2 antagonist, are interesting considering the fact that repeated treat-

ment with amantadine induces a moderate increase in [^{11}C]-raclopride binding, indicating an increase of D2 receptors in patients with Parkinson's disease.⁴⁵

Finally, the overall correlation between apparent functional (Ca^{2+} assay) and radioligand binding (literature) K values is quite good (**Figure 4A, 4B**). There is a significant correlation of apparent functional and binding K values at D1 and D2 receptors ($P < .0001$), yielding r^2 values of 0.7796 and 0.7743, respectively. However, for some compounds there is a discrepancy between apparent functional and binding K values of up to 10-fold: fluphenazine at D2 receptors does not fit into the rank order of potency (**Table 2**). The apparent functional K value is ~10-fold too high. However, it is the only compound at D2 receptors that does not fit into the rank order of potency. At D1 receptors, apparent functional K values are generally higher than their corresponding literature-based radioligand binding K values (**Table 1**). Only chlorpromazine and lisuride do not fit into the rank order of potency (measured as apparent functional K). However, the deviation in the rank order of potency is small for chlorpromazine, and radioligand binding K values for lisuride were measured at calf or dog striatum, thus possibly adding to the difference between functional and binding data. When comparing apparent functional with binding K values, one also has to take into account that apparent K values depend on the expression level of the respective receptor. Thus, the expression level of D1 and D2 receptors together with the used agonist concentration in the Ca^{2+} assay will influence the apparent functional K and lead to different values than binding K's. This — at first view — limitation of the functional Ca^{2+} assay is no problem if test compounds are measured together with known (standard) ligands under exactly the same experimental conditions, and the rank order of potency of all compounds is subsequently compared. Looking at the rank order of potency, only 1 or 2 compounds out of 17 at the D2 or the D1 receptor did not fit into the rank order (**Tables 1 and 2**). Further, it needs to be taken into account that radioligand binding data in the literature are not too consistent. For haloperidol, for instance, K values between 0.35 and 9.6 nM were found for D2 receptors.^{25,26} Some of these variations may be explained by different experimental setups: not all radioligand binding K values in the literature were obtained at human receptors (**Tables 1 and 2**). Further, some experiments were performed with recombinantly expressed receptors, some with native tissue (**Tables 1 and 2**, "Binding K Literature"). Another issue is that radioligand binding experiments are performed in equilibrium. In the Ca^{2+} assay, the antagonist is allowed to equilibrate with the receptor, but subsequent injection of agonist induces a fast signal (peaking within ~15 seconds, **Figure 3A**) not allowing equilibrium between antagonist, agonist, and receptor, and thus — depending on the dissociation rate constant of the antagonist — leading to decreased or increased apparent functional K values.

Apparent functional K values obtained with the Ca^{2+} assay are also in accordance with functional data in the lit-

erature measured by [^{35}S]GTPγS binding.⁴⁶ Gilliland and Alper found the same rank order of potency for agonists and antagonists at D2 receptors as is presented in **Table 2** in apparent functional K values (agonists: pergolide>apomorphine>quinpirole; antagonists: spiperone>haloperidol>clozapine). In conclusion, considering the differences in experimental setups (buffers, source of receptors, equilibrium or nonequilibrium conditions), the overall correlation between apparent functional K values obtained by the Ca^{2+} assay and literature-based radioligand binding K values is significant for D1 and D2 receptors (**Figure 4**). Further, the rank order of potency remains the same for both functional and radioligand binding data with few exceptions (**Tables 1 and 2**). Taken together, these results suggest that screening of potential ligands at dopamine receptors with the simple, medium-throughput functional Ca^{2+} assay as recently described¹¹ may allow the replacement of radioligand binding experiments, and additionally yield information about agonistic or antagonistic behavior of a compound.

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