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European Journal of Pharmacology 540 (2006) 53–56
www.elsevier.com/locate/ejphar

Short communication

In vitro biochemical evidence that the psychotomimetics phencyclidine, ketamine and dizocilpine (MK-801) are inactive at cloned human and rat dopamine D_2 receptors

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Received 21 March 2006; received in revised form 21 April 2006; accepted 25 April 2006
Available online 3 May 2006

Abstract

Dopamine potently increased calcium mobilization in Chinese hamster ovary cells expressing human dopamine D_{2Long} receptors (CHO-D_{2L} cells), and increased guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate binding to CHO-D_{2L} cell and rat striatal membranes. These effects of dopamine were blocked by the dopamine D_2 receptor antagonist (−)raclopride. In contrast to the findings of a recent controversial study, phencyclidine, ketamine and dizocilpine (MK-801) lacked dopamine D_2 receptor full agonist, partial agonist and antagonist activity in these assays, suggesting their psychotomimetic effects, and activity in rodent models of schizophrenia, are associated with *N*-methyl-D-aspartate receptor blockade rather than a direct interaction with dopamine D_2 receptors.

Keywords: Schizophrenia; Glutamate; Antipsychotic; Confocal microscopy

1. Introduction

The dopamine hyperfunction hypothesis of schizophrenia is supported by the clinical efficacy of first generation antipsychotic drugs, such as chlorpromazine and haloperidol, being a positive correlate of their potency as dopamine D_2 receptor antagonists (Seeman et al., 1976), and by observations that increases in extracellular dopamine accompany amphetamine-induced psychosis (Laruelle et al., 1996). More recently, however, the glutamate/*N*-methyl-D-aspartate (NMDA) receptor hypothesis of schizophrenia has been formulated, on the basis that psychotomimetic concentrations of phencyclidine (PCP), ketamine and dizocilpine (MK-801) block NMDA receptors (Chavez-Noriega et al., 2005). Nevertheless, these drugs bind with higher affinity to a high affinity state of cloned human dopamine D_{2Long} (D_{2L}) receptors (PCP, K_i=2.7 nM; ketamine, K_i=55 nM; MK-801, K_i=0.3 nM), expressed in the presence of a subphysiological (10 mM) concentration of NaCl, than to cloned human NMDA receptors (PCP, K_i=196 nM; ketamine, K_i=3.15 μM; MK-801, K_i=3 nM) (Seeman et al., 2005). Furthermore, these drugs behaved as dopamine D_{2L} receptor full agonists (PCP, EC_{50}=90 nM; ketamine, EC_{50}=110 nM; MK-801, EC_{50}=8 nM) in a guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding assay using Chinese hamster ovary (CHO) cell homogenates expressing human D_{2L} receptors in the presence of 10 mM NaCl (Seeman et al., 2005) and, in an earlier study, these drugs exhibited human dopamine D_{2L} receptor partial agonist activity in the same assay (Kapur and Seeman, 2002). These in vitro observations that PCP, ketamine and MK-801 bind with high affinity and behave as human dopamine D_{2L} receptor agonists predict a more prominent role for dopamine D_2 receptors, compared with NMDA receptors, in mediating the psychotomimetic effects of these drugs in humans and their activities in rodent models of schizophrenia, although these data are currently controversial (Svenningsson et al., 2004).

This study estimated the in vitro potency and relative intrinsic activity of PCP, ketamine and MK-801 at human dopamine D_{2L} receptors, with respect to their effects on [^{35}S]GTPγS binding to CHO-D_{2L} cell membranes and calcium (Ca^{2+}) mobilization in

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doi:10.1016/j.ejphar.2006.04.026

CHO-D_{2L} cells. We also investigated the effects of these drugs on [^{35}S]GTPγS binding to membranes from rat dorsal striatum, a brain region populated with high and low densities of dopamine D_2 and D_3 receptors, respectively (Missale et al., 1998).

2. Materials and methods

[^{35}S]GTPγS (1200 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). PCP, ketamine, MK-801, dopamine, (+)terguride, *S*(−)-3-(3-hydroxyphenyl)-*N*-*n*-propylpiperidine ((−)3-PPP), *R*(−)-propylnorapomorphine ((−)NPA) and GDP were obtained from Sigma Chemical Company (St. Louis, MO). All cell culture reagents, Dulbecco's phosphate buffered saline (DPBS), Pluronic F-127 and Fluo-3AM were purchased from Invitrogen Corp. (Carlsbad, CA).

The potency (pEC_{50}) and relative intrinsic activity (E_{max}, maximal drug effect, expressed as a percentage of the effect of 10 μM dopamine (human dopamine D_{2L} receptor assays) and 10 μM (−)NPA (rat striatal membrane assay)) were estimated by testing dopamine, (−)3-PPP, (+)terguride, PCP, ketamine, MK-801 and (−)raclopride in triplicate at 8 to 10 different concentrations in each assay.

The [^{35}S]GTPγS binding assays were performed by incubating vehicle and each drug for 40 min at 22 °C with CHO-D_{2L} cell membranes (40 μg protein), mixed with buffer (20 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, pH=7.4) containing GDP (1.65 μM) and [^{35}S]GTPγS (30 pM). 10 μM concentrations each of dopamine, PCP, ketamine and MK-801 were also tested in this assay using a low NaCl concentration (10 mM). In a separate assay, vehicle and each drug were incubated for 60 min at 30 °C with rat striatal membranes (20 μg protein), mixed with buffer (50 mM Tris HCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA and 0.1% sodium metabisulphite, pH=7.4) containing GDP (100 μM) and [^{35}S]GTPγS (100 pM). All [^{35}S]GTPγS assay reactions were terminated by rapid filtration through Whatman GF/B filter paper presoaked in 50 mM Tris HCl, pH=7.4, using a Brandel harvester and 4×3 ml ice-cold washes with the same buffer, and [^{35}S] radioactivity bound to each filter paper was detected by liquid scintillation counting. CHO-D_{2L} cell and rat striatal membranes were generated using published methods for preparing recombinant cell and rat brain membranes (Jordan et al., 2005). The same [^{35}S]GTPγS binding assays were also used to estimate the inhibitory potency (pIC_{50}) of (−)raclopride, PCP, ketamine and MK-801, each tested at eight different concentrations ranging from 0.01 nM to 10 μM, against 1 μM dopamine at human dopamine D_{2L} receptors and rat striatal membranes.

For the Ca^{2+} mobilization assay, CHO-D_{2L} cells were plated (300,000 cells/ml) in Nunc Lab-Tek II chambered slides and incubated at 37 °C for ~ 24 h. These cells were then washed twice with DPBS, incubated under aluminum foil for 75 min in fluorescent loading solution (4 μM Fluo-3AM and 0.066% Pluronic F-127 diluted in DPBS), washed twice with DPBS and then incubated in darkness for 30 min. Digital images of fluorescently labeled intracellular Ca^{2+} were acquired at 5-s intervals, before and after the cells were exposed to vehicle or drug, using a confocal laser-scanning microscope (488 nM argon laser using LP505 emission filter and X20 lens magnification). Vehicle and drug effects on intracellular Ca^{2+} mobilization were quantitated using calibrated densitometric image analysis of the mean fluorescence intensity in each image. This protocol was also used to study the effects of 10 μM (−)raclopride, PCP, ketamine and MK-801 on dopamine (1 μM)-induced Ca^{2+} mobilization in CHO-D_{2L} cells.

All estimates of potency, relative intrinsic activity and inhibitory potency were calculated by non-linear regression analysis of each binding isotherm using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

3. Results

Dopamine behaved as a human dopamine D_{2L} receptor full agonist, with respect to its weakly potent stimulation of [^{35}S]GTPγS binding to CHO-D_{2L} cell membranes (pEC_{50}=6.0±0.08) and potent mobilization of Ca^{2+} in CHO-D_{2L} cells (pEC_{50}=8.4±0.08). In comparison, (+)terguride and (−)3-PPP exhibited human dopamine D_{2L} receptor partial agonist activity in these assays, and these drugs both displayed a 6-fold higher relative intrinsic activity in the Ca^{2+} assay ((+)terguride,

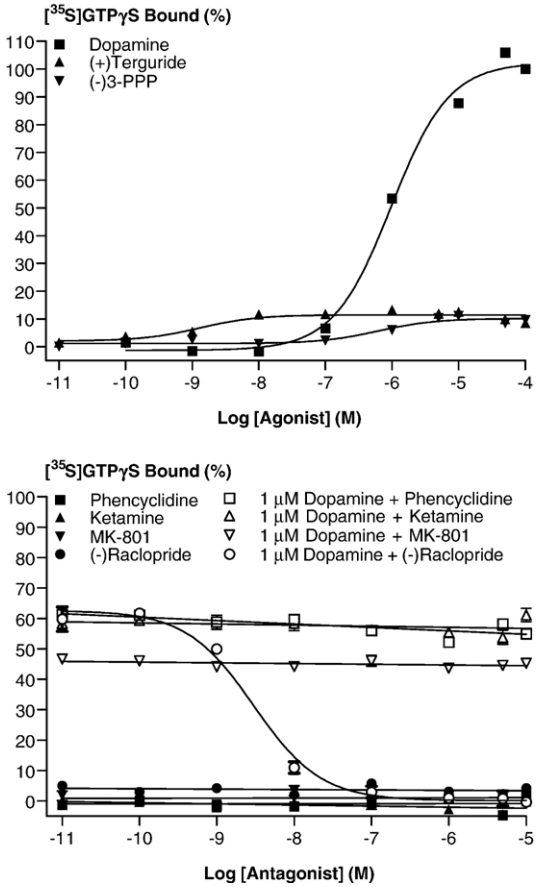

Fig. 1. Drug effects on [^{35}S]GTPγS binding to CHO-D_{2L} cell membranes in the presence of 150 mM NaCl. Data points are means±S.E.M. of pooled data from three representative experiments, each containing triplicate reactions, and are expressed as a percentage of the effect of 10 μM dopamine on [^{35}S]GTPγS binding.

Table 1
Functional parameter estimates for the effects of psychotomimetic and reference dopaminergic drugs on [^{35}S]GTPγS binding to membranes from CHO-D_{2L} cells and rat striatum, both performed in the presence of 150 mM NaCl, and their effects on Ca^{2+} mobilization in CHO-D_{2L} cells

	CHO-D_{2L} [^{35}S]GTPγS		CHO-D_{2L} Ca^{2+}		Rat striatum [^{35}S]GTPγS	
Agonist	pEC_{50}±S.E.M.	E_{max} (%)	pEC_{50}±S.E.M.	E_{max} (%)	pEC_{50}±S.E.M.	E_{max} (%)
Dopamine	6.0±0.08	102.3	8.4±0.25	101.3	5.48±0.16	103.7
(+)Terguride	8.9±0.34	11.5	7.6±0.08	72.9	Inactive	
(−)3-PPP	6.2±0.25	10.2	6.8±0.19	64.7	Inactive	
	CHO-D_{2L} [^{35}S]GTPγS		CHO-D_{2L} Ca^{2+}		Rat striatum [^{35}S]GTPγS	
Inhibition	pIC_{50}±S.E.M.		Effect on 1 μM dopamine		pIC_{50}±S.E.M.	
(−)Raclopride	8.52±0.08		Blocked		9.09±0.19	

Agonist potency (pEC_{50}), relative intrinsic activity (E_{max}), maximal drug effect expressed as a percentage of 10 μM dopamine or 10 μM (−)NPA (rat striatum assay only) were estimated by non-linear regression analysis of the concentration–effect data for each drug. Non-linear regression was also used to estimate the inhibitory potency (pIC_{50}) of (−)raclopride in both [^{35}S]GTPγS binding assays against 1 μM dopamine. PCP, ketamine, MK-801 and (−)raclopride were inactive as agonists in all three assays, and (−)raclopride was the only of these compounds to produce antagonist activity in the same assays. Dopamine (1 μM)-induced Ca^{2+} mobilization was blocked by 10 μM (−)raclopride but not by the same concentration of PCP, ketamine or MK-801. All values represent means±S.E.M. of three representative experiments each containing triplicate reactions.

E_{max}=72.9% of 10 μM dopamine; (−)3-PPP=64.7%). In contrast, [^{35}S]GTPγS binding and Ca^{2+} mobilization were unaffected by PCP, ketamine, MK-801 and (−)raclopride. However, (−)raclopride blocked 1 μM dopamine activity in both the [^{35}S]GTPγS binding and Ca^{2+} assay, although dopamine activity was not affected by PCP, ketamine or MK-801. These drug effects on [^{35}S]GTPγS binding to CHO-D_{2L} cell membranes, observed in the presence of 150 mM NaCl, are shown in Fig. 1. Dopamine also increased [^{35}S]GTPγS binding to CHO-D_{2L} cell membranes under conditions of low NaCl (10 mM), while PCP, ketamine and MK-801 were inactive in this respect (mean counts/min±S.E.M., vehicle=13,098±14, 10 μM dopamine=21,448±51.4, 10 μM PCP=13,344±335, 10 μM ketamine=12,957±213, 10 μM MK-801=12,786±79). Dopamine increased [^{35}S]GTPγS binding to rat striatal membranes with weak potency (pEC_{50}=5.48±0.15), in contrast to the inactivity of (−)terguride, (−)3-PPP, PCP, ketamine, MK-801 and (−)raclopride. Dopamine (1 μM) activity in this assay was potently blocked by (−)raclopride, although dopamine activity was unchanged in response to a single concentration (10 μM) of PCP, ketamine and MK-801. Table 1 lists the in vitro functional characteristics of all drugs tested in these assays.

4. Discussion

PCP, ketamine and MK-801 have each demonstrated human dopamine D_{2L} receptor full agonist and partial agonist activities in a [^{35}S]GTPγS binding assay performed using CHO-D_{2L} cell homogenates in the presence of 10 mM NaCl (Kapur and Seeman, 2002; Seeman et al., 2005). However, neither of these drugs displayed human dopamine D_{2L} receptor full agonist, partial agonist or antagonist activities either in our CHO-D_{2L} cell membrane [^{35}S]GTPγS binding assay, conducted in the presence of 10 mM and 150 mM NaCl, or CHO-D_{2L} cell Ca^{2+} mobilization assay. These data argue against a role for human dopamine D_2 receptors in mediating the psychotomimetic properties of PCP, ketamine and MK-801. In addition, these drugs did not affect basal or dopamine-induced [^{35}S]GTPγS binding to rat striatal membranes, which suggests the activities of PCP, ketamine and MK-801 in rodent models of schizophrenia are more likely associated with their blockade of NMDA receptors than a direct interaction with dopamine D_2 receptors. The current assays served as reliable readouts of drug activity at human dopamine D_{2L} receptors and striatal dopamine D_2/D_3 receptors, as dopamine (1 μM) activity was potently blocked in every assay by the dopamine D_2/D_3 receptor antagonist (−) raclopride. Furthermore, our estimates of potency and relative intrinsic activity for dopamine, (+)terguride and (−)3-PPP are similar to previously published estimates for these drugs at human dopamine D_{2L} receptors and in rat striatal membrane [^{35}S]GTPγS binding assays (Gardner and Strange, 1998; Geurts et al., 1999; Jordan et al., 2004; Rinken et al., 1999).

Dopamine, PCP, ketamine and MK-801 bind with high affinity to a high affinity state of human dopamine D_{2L} receptors (D_{2L} high) expressed in the presence of 10 mM NaCl (Seeman et al., 2003, 2005). The low nanomolar potency of dopamine (EC_{50}=4.0 nM) in our Ca^{2+} mobilization assay is very close to its binding affinity (K_i=1.75 nM) at human dopamine D_{2L} high receptors (Seeman et al., 2003), suggesting the inactivity of PCP, ketamine and MK-801 in our Ca^{2+} assay is not related to a low level of human dopamine D_{2L} high receptor expression in our CHO-D_{2L} cells. On the other hand, the 250-fold lower potency of dopamine in our [^{35}S]GTPγS binding assay is consistent with a comparatively lower density of human dopamine D_{2L} high receptors being expressed in our CHO-D_{2L} membranes. It must also be remembered, however, that [^{35}S]GTPγS binding assays typically yield lower estimates of agonist potency and relative intrinsic activity, as they provide a more upstream readout of dopamine D_2 receptor signal transduction that is less amplified and subject to regulation by other cellular processes, compared with Ca^{2+} mobilization and other assays that provide more downstream signaling readouts (Milligan, 2003). Lower estimates of agonist potency and relative intrinsic activity in [^{35}S]GTPγS binding assays might also be attributed to the use of GDP in the assay buffer, which is

typically required to achieve optimal assay sensitivity and dynamic range, albeit at the expense of uncoupling dopamine D_2 receptors from their cognate G proteins (Roberts et al., 2004).

In the rat striatal membrane assay, dopamine was weakly potent and (+)terguride and (−)3-PPP were inactive, although these latter compounds are active at rat dopamine D_2 receptors in vivo (Missale et al., 1998). These data probably reflect the high GDP concentration and low level of striatal dopamine D_2 receptor expression (B_{max} =212 fmol/mg protein—data not shown) in our assay, as lower estimates of agonist potency and relative intrinsic activity occur with reductions in receptor expression density. Furthermore, these effects of GDP and receptor expression density are more noticeable for partial than full agonist drugs (Hoyer and Boddeke, 1993; Roberts et al., 2004).

Together, these data are supportive of clinical and preclinical evidence, as discussed by Svenningsson et al. (2004), that dopamine D_2 receptors do not play a major role in mediating the psychotomimetic activity of PCP, ketamine and MK-801 in humans or the activity of these drugs in rodent models of schizophrenia used to detect antipsychotic drug activity.

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