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G Protein Activation by Human Dopamine D_3 Receptors in High-Expressing Chinese Hamster Ovary Cells: A Guanosine-5′-*O*-(3-[^{35}S]thio)-Triphosphate Binding and Antibody Study

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ABSTRACT

Despite extensive study, the G protein coupling of dopamine D_3 receptors is poorly understood. In this study, we used guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]-GTPγS) binding to investigate the activation of G proteins coupled to human (h) D_3 receptors stably expressed in Chinese hamster ovary (CHO) cells. Although the receptor expression level was high (15 pmol/mg), dopamine only stimulated G protein activation by 1.6-fold. This was despite the presence of marked receptor reserve for dopamine, as revealed by Furchgott analysis after irreversible hD_3 receptor inactivation with the alkylating agent, EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline). Thus, half-maximal stimulation of [^{35}S]-GTPγS binding required only 11.8% receptor occupation of hD_3 sites. In contrast, although the $hD_{2(short)}$ receptor expression level in another CHO cell line was 11-fold lower, stimulation by dopamine was higher (2.5-fold). G protein activation was increased at hD_3 and, less potently, at hD_2 receptors by the preferential D_3 agonists, PD 128,907 [(+)-(4aR,10bR)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H- [1]benzopyrano[4,3-b]-1,4-oxazin-9-ol] and (+)-7-OH-DPAT (7-hydroxy-2-(di-*n*-propylamino)tetralin). Furthermore, the selective D_3 antagonists, S 14297 ((+)-[7-(*N*, *N*-dipropylamino)-5,6,7,8-tetrahydro-naphtho(2,3b)dihydro-2,3-furane]) and GR 218,231 (2(*R*,*S*)-(dipropylamino)-6-(4-methoxyphenylsulfonylmethyl)-1,2,3,4- tetrahydronaphtalene), blocked dopamine-stimulated [^{35}S]GTPγS binding more potently at hD_3 than at hD_2 sites. Antibodies against $G\alpha_i/\alpha_o$ reduced dopamine-induced G protein activation at both CHO-hD_3 and -hD_2 membranes, whereas $G\alpha_S$ antibodies had no effect at either site. In contrast, incubation with anti-$G\alpha_q/\alpha_{11}$ antibodies, which did not affect dopamine-induced G protein activation at hD_2 receptors, attenuated hD_3-induced G protein activation. These data suggest that hD_3 receptors may couple to $G\alpha_q/\alpha_{11}$ and would be consistent with the observation that pertussis toxin pretreatment, which inactivates only $G_{i/o}$ proteins, only submaximally (80%) blocked dopamine-stimulated [^{35}S]GTPγS binding in CHO-hD_3 cells. Taken together, the present data indicate that 1) hD_3 receptors functionally couple to G protein activation in CHO cells, 2) hD_3 receptors activate G proteins less effectively than hD_2 receptors, and 3) hD_3 receptors may couple to different G protein subtypes than hD_2 receptors, including non-pertussis sensitive $G_{q/11}$ proteins.

Dopaminergic neurotransmission is mediated by five receptor subtypes (D_1 to D_5) which can be grouped into two receptor families. D_1-like receptors include the D_1 and D_5 subtypes, whereas D_2-like receptors include the D_2, D_3, and D_4 subtypes. D_2 and D_3 receptors, in particular, display marked sequence homology and pharmacological similarity in their in vitro ligand binding profiles (Levant, 1997; Missale et al., 1998). However, D_3 receptors may be distinguished from D_2 receptors by several factors. D_3 receptors are concentrated in limbic rather than striatal brain regions (Liu et al., 1996; Hall et al., 1996). Furthermore, they mediate stimulation, rather than inhibition, of c-*fos* expression in striatal neurones (Pilon et al., 1994; Morris et al., 1997), and inhibition, rather than stimulation, of locomotor activity in rats (Svensson et al., 1994; Starr and Starr, 1995). In addition, whereas D_2 receptors couple efficiently to second-messenger systems, markedly inhibiting adenylyl cyclase activity, such responses have proved elusive and complex for D_3 receptors (e.g., Freedman et al., 1994; MacKenzie et al., 1994; Tang et al., 1994; Griffon et al., 1997). Indeed, D_3 receptors couple selectively to inhibition of adenylyl cyclase type V, but not type I or VI, and only weakly to type II (Robinson and

[1] These two authors made equivalent contributions to this work.

ABBREVIATIONS: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; GR 218,231, 2(*R*,*S*)-(dipropylamino)-6-(4-methoxyphenylsulfonylmethyl)-1,2,3,4- tetrahydronaphtalene; [^{35}S]GTPγS, guanosine-5′-O-(3-[^{35}S]thio)-triphosphate; (+)7-OH-DPAT, 7-hydroxy-2-(di-*n*-propylamino)-tetralin; PD 128,907, (+)-(4aR,10bR)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-[1]benzopyrano[4,3-b]-1,4-oxazin-9-ol; S 14297, (+)-[7-(N, N-dipropylamino)-5,6,7,8-tetrahydro-naphtho(2,3b)dihydro-2,3-furane].

Caron, 1997; Watts and Neve, 1997). In vitro studies of agonist efficacy have employed other measures of receptor activation, including medium acification (Cox et al., 1995), and stimulation of mitogenesis (Pilon et al., 1994; Svensson et al., 1994; Sautel et al., 1995). However, these approaches measure responses "downstream" of the receptor in the intracellular activation cascade and the relevance of an increase in mitogenesis for postmitotic central nervous system neurones is unclear. A more promising approach may be to measure receptor-mediated G protein activation by stimulation of guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding: this corresponds to the first step of the intracellular activation cascade and directly reflects ligand binding events at the receptor itself (Pregenzer et al., 1997; Malmberg et al., 1998). Thus, the present study adopted this strategy to address several questions concerning, principally, the functional properties of human (h) D_3 receptors. In addition, in some tests results at hD_3 receptors were compared with those at hD_2 receptors. First, differences in the second-messenger actions of D_3 and D_2 receptors may be related to differing capacities for stimulation of G proteins. We addressed this issue by investigating the ability of hD_3 receptors to mediate dopamine-stimulated [^{35}S]GTPγS binding. Second, the relationship between binding affinity and functional potency of dopaminergic agonists and antagonists was investigated using the most potent and selective D_3 receptor ligands reported to date: the agonists (+)-7-OH-DPAT (7-hydroxy-2-(di-*n*-propylamino)tetralin) and PD 128,907 [(+)-(4aR,10bR)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H- [1]benzopyrano[4,3-b]-1,4-oxazin-9-ol] (Pugsley et al., 1995) and the antagonists, S 14297 ((+)-[7-(N, N-dipropylamino)-5,6,7,8-tetrahydro-naphtho(2,3b)dihydro-2,3-furane]) and GR 218,231 (2(*R,S*)-(dipropylamino)-6-(4-methoxyphenylsulfonylmethyl)-1,2,3,4-tetrahydronaphtalene) (Millan et al., 1995b; Murray et al., 1996). The hD_3/hD_2 selectivities based on K_i ratios were compared with those based on EC_{50} and K_B ratios (Burris et al., 1995; Levant, 1997). Third, the signal transduction differences between D_3 and D_2 receptors, such as the differential coupling to adenylyl cyclase isoforms, could be due to receptor interactions with different G protein populations. Indeed, at least 16 distinct G protein α subunits have been identified, divided into four families: G_i, G_S, $G_{q/11}$, and $G_{12/13}$ (Simon et al., 1991). Although a previous study suggested differences in coupling profiles of D_2 and D_3 receptors for modulation of outward K^+ currents (Liu et al., 1996), no information is available from a functional test more proximal to the receptor and the G protein subtypes involved in D_3 coupling are unclear (cf. Tang et al., 1994). The present study, therefore, examined G protein coupling specificity directly at the G protein activation level by challenging the receptor-mediated stimulation of [^{35}S]GTPγS binding with specific antisera raised against different Gα subunits. In fact, antibodies raised against the COOH terminal part of Gα subunits have proved useful to determine the G protein specificity of several other 7-transmembrane domain receptors (Harris-Warrick et al., 1988; McFadzean et al., 1989; Lledo et al., 1992; Izenwasser and Côté, 1995).

TABLE 1

Densities of recombinant receptors and agonist-activated G proteins in CHO cells stably expressing hD_2 and hD_3 receptors

Receptor expression levels (B_{max}) of hD_3 and hD_2 receptors stably expressed in CHO cell membranes were determined by saturation binding experiments with [^{125}I]iodosulpride. Treatment of CHO-hD_3 membranes with EEDQ (33 μM) significantly reduced hD_3 receptor expression ($p < 0.05$, 2-tailed t test). Number of dopamine-activated G proteins was determined by [^{35}S]GTPγS isotopic dilution saturation binding, as described in *Materials and Methods*. Apparent K_D for [^{35}S]GTPγS saturation binding is denoted K_{APP}. EEDQ (33 μM) treatment of CHO-hD_3 membranes did not significantly alter B_{max} or K_{APP} for [^{35}S]GTPγS saturation.

Cell Line	CHO-hD_3	CHO-hD_3 (EEDQ)	CHO-hD_2
Receptor Saturation			
B_{max} (pmol/mg)	15.43 ± 1.33	7.36 ± 1.17	1.39 ± 0.19
K_D (nM)	1.18 ± 0.19	1.31 ± 0.03	0.48 ± 0.05
G Protein Saturation			
B_{max} (pmol/mg)	3.38 ± 0.51	2.58 ± 0.53	0.92 ± 0.05
K_{APP} (nM)	6.72 ± 1.29	6.18 ± 1.08	1.68 ± 0.30
R/G B_{max} Ratio	4.6	2.9	1.5

Data are means ± S.E.M. of at least three independent determinations.

Materials and Methods

Membrane Preparations of Chinese Hamster Ovary (CHO)-hD_3 and CHO-hD_2 Cells. CHO cells expressing hD_3 receptors were grown as described previously (Sokoloff et al., 1992). Cells were harvested from adherent culture and homogenized using a Kinematica Polytron (Kinematica GmbH, Littau, Switzerland) in a buffer containing 50 mM Tris (pH 7.4), 5 mM $MgCl_2$. The suspension was then centrifuged at 20,000*g* for 15 min at 4°C and the pellet was resuspended in the appropriate binding buffer (see below) and stored

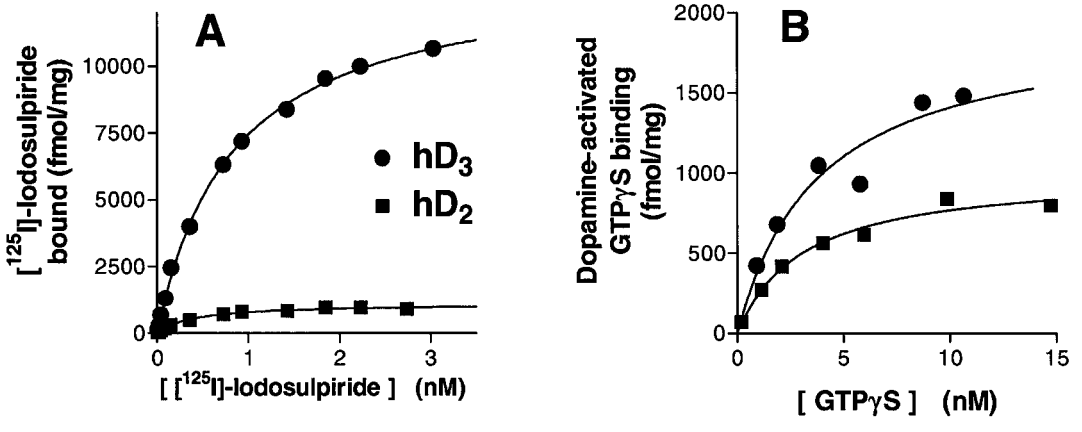

Fig. 1. Saturation binding of [^{125}I]iodosulpride and [^{35}S]GTPγS to CHO-hD3 and CHO-hD_2 cell membranes. A, representative saturation binding isotherms of [^{125}I]iodosulpride to CHO-hD_2 and CHO-hD_3 membranes. B, representative saturation binding isotherms of [^{35}S]GTPγS to CHO-hD_2 and CHO-hD_3 membranes. Basal and dopamine (10 μM)-stimulated [^{35}S]GTPγS binding were determined in the presence of increasing concentrations of GTPγS. These data were transformed as described in *Materials and Methods* to generate a saturation binding isotherm for net agonist-dependent [^{35}S]GTPγS binding. Points shown are means of duplicate determinations from representative experiments repeated on at least four occasions. B_{max} and K_D/apparent K_D (K_{app}) data from these experiments are shown in Table 1.

at −80°C. CHO-$hD_{2(short)}$ cell membranes were purchased from Receptor Biology (Baltimore, MD). The "short" hD_2 isoform, which lacks a 29-amino acid insert in the putative third intracellular loop, is processed faster to mature receptors at the cell surface than the "long" form and may couple more efficiently to certain G protein subtypes (Fishburn et al., 1995; Boundy et al., 1996).

[^{125}I]Iodosulpride Binding to hD_3 and hD_2 Receptors. Saturation binding at hD_2 and hD_3 receptors was carried out with 12 concentrations of [^{125}I]iodosulpride (1000 Ci/mmol; Amersham, Les Ulis, France). For competition binding experiments, membranes (10 to 20 μg protein) of CHO-hD_2 or CHO-hD_3 cells were incubated with [^{125}I]iodosulpride (0.1 nM for hD_2 and 0.2 nM for hD_3) at 30°C for 30 min in a buffer containing 50 mM Tris (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM EDTA, and 5 mM $MgCl_2$. Nonspecific binding was defined with raclopride (10 μM). Isotherms were analyzed by nonlinear regression, using the computer program PRISM (Graphpad Software Inc., San Diego, CA) to yield IC_{50} values. Inhibition constants (K_i values) were derived from IC_{50} values according to the Cheng-Prusoff equation. The goodness of fit was tested by runs test. For compounds that yielded $P < .05$ in the runs test and/or shallow inhibition isotherms (nH values markedly inferior to unity), 14-point competition binding experiments were carried out and one- and two-site fits were compared by F test.

Measurement of Agonist Efficacy and Antagonist Potency at hD_3 and hD_2 Receptors. Receptor-linked G protein activation by dopamine at hD_2 and hD_3 receptors was determined by measuring the stimulation of [^{35}S]GTPγS (1332 Ci/mmol; NEN, Les Ulis, France) binding induced by dopamine. CHO-hD_2 membranes (30–40 μg protein) were incubated (60 min, 22°C) with agonists and/or antagonists in a buffer containing 20 mM HEPES (pH 7.4), 3 μM GDP, 10 mM $MgCl_2$, 150 mM NaCl, and 0.1 nM [^{35}S]GTPγS. CHO-hD_3 membranes (30–50 μg protein) were incubated (40 min, 22°C) with agonists and/or antagonists in a buffer containing 20 mM HEPES (pH 7.4), 3 μM GDP, 3 mM $MgCl_2$, 100 mM NaCl, and 1.0 nM [^{35}S]GTPγS. Nonspecific binding was defined with GTPγS (10 μM). Agonist efficacy is expressed relative to that of dopamine (100%), which was tested at a maximally effective concentration (10 μM) in each experiment. For all tests, membranes were preincubated with agonist and/or antagonist for 15 min before the addition of [^{35}S]GTPγS. K_B values for inhibition of dopamine (1 and 3 μM for hD_3 and hD_2 respectively)-stimulated [^{35}S]GTPγS binding were calculated according to Lazareno and Birdsall (1993):

$$K_B = IC_{50}/\{[(2+(\text{agonist}/EC_{50})^{nH})^{nH^{-1}}] - 1\};$$

where IC_{50} is the inhibitory concentration$_{50}$ of the antagonist, agonist is the dopamine concentration, EC_{50} is the effective concentration$_{50}$ of dopamine alone, and nH is the Hill coefficient of the dopamine stimulation isotherm.

For dopamine concentration-response curves determined in the presence of fixed concentrations of the antagonist, GR 218,231, pA_2 values were derived by Schild analysis. In isotopic dilution experiments, the basal and dopamine (10 μM)-stimulated binding of radiolabeled [^{35}S]GTPγS was inhibited with unlabeled GTPγS. Saturation binding curves were derived to estimate the number of G proteins activated by dopamine, as described previously (Newman-Tancredi et al., 1997).

Experiments were terminated by rapid filtration through Whatman GF/B filters (pretreated with 0.1% polyethyleneimine in the case of [^{125}I]iodosulpride binding) using a Brandel cell harvester. Radioactivity retained on the filters was determined by liquid scintillation counting. All data are expressed as mean ± S.E.M. of ≥3 independent determinations. Protein concentration was determined colorimetrically using a bicinchonic acid assay kit (Sigma Chemical Co., S. Quentin Fallavier, France).

hD_3 Receptor Alkylation with *N*-Ethoxycarbonyl-2-Ethoxy-1,2-Dihydroquinoline (EEDQ). CHO-hD_3 cell membranes were treated by EEDQ at a final concentration between 0 and 300 μM (0, 10, 33, 100, and 300 μM). The membrane suspension (3 ml final

TABLE 2

Concentration-dependent actions of EEDQ on receptor density and function in CHO-hD_3 membranes

CHO-hD_3 cell membranes were treated with different concentrations of EEDQ. Receptor density was determined in [^{125}I]iodosulpiride saturation binding experiments. Percentage of remaining hD_3 binding sites is calculated as a percentage of mean B_{max} determined in absence of EEDQ (15.43 pmol/mg, Table 1). [^{35}S]GTPγS binding is expressed as percentage of dopamine (10 μM)-dependent binding observed under control conditions in parallel experiments.

EEDQ	Receptor Saturation		[^{35}S]GTPγS Bound
	K_D	B_{max}	
μM	*nM*	*% of control*	*% of control*
0	1.18 ± 0.19	100	100
10	1.38 ± 0.01	71.5 ± 9.3	77.5 ± 8.7
33	1.31 ± 0.03	47.7 ± 7.6	51.2 ± 1.6
100	1.24 ± 0.11	32.0 ± 4.7	44.9 ± 2.0
300	1.33 ± 0.09	18.7 ± 6.4	33.6 ± 5.6

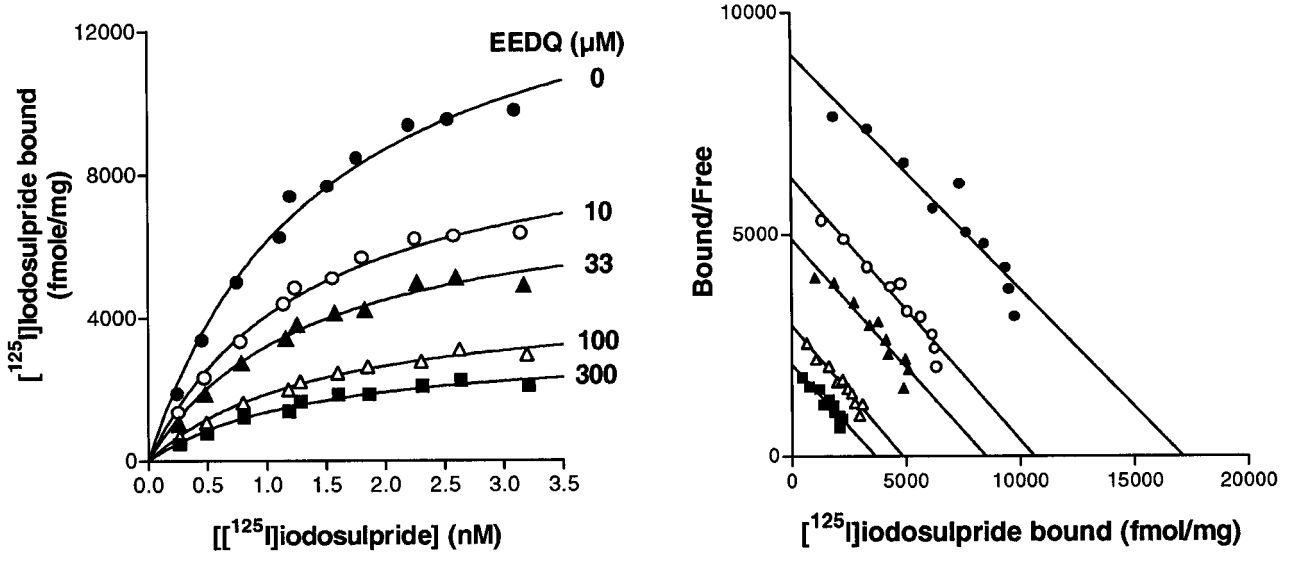

Fig. 2. Concentration-dependent reduction of hD_3 receptor density by EEDQ. A, representative saturation binding isotherms of [^{125}I]iodosulpride to CHO-hD_3 membranes pretreated with different concentrations of EEDQ. B, Scatchard representation of data from A. Points shown are means of duplicate determinations from representative experiments repeated on at least three occasions. B_{max} and K_D data from these experiments are shown in Table 2.

volume) was vortexed and immediately centrifuged at 4°C for 15 min at 20,000*g*. The supernatant was discarded and the membrane pellet was resuspended in the appropriate buffer and [^{35}S]GTPγS or [^{125}I]iodosulpride binding was performed as described above. K_A values were determined by Furchgott analysis, as described by Atkinson and Minneman (1992) and Adham et al. (1993), with CHO-hD_3 membranes treated with 33 μM EEDQ. Plots were derived of 1/[A] versus 1/[A′]; where [A] and [A′] are equiactive concentrations for stimulation of [^{35}S]GTPγS binding before and after receptor alkylation, respectively. K_A was calculated from

K_A = (slope-1)/y-intercept.

Percentage receptor occupancy (O) was calculated by

$O = 100 * L/(L + K_A)$;

where L is the concentration of agonist.

Characterization of G proteins by Immunoblotting and ADP-Ribosylation. Immunoblotting of Gα subunits was performed using antisera purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) raised against $G\alpha_{i/O}$ (C10), $G\alpha_S$ (C18), and $G\alpha_{q/11}$ (C19). Approximately 2 μg protein from CHO-hD_2 and CHO-hD_3 membrane preparation was separated on 10% polyacrylamide gel and transferred onto nitrocellulose. Antisera were incubated at 1/1000 followed by enhanced chemiluminescence detection with horseradish peroxidase as secondary antibody (Amersham, Buckinghamshire, UK).

ADP-ribosylation by *Bordetella pertussis* toxin (PTX) was carried out as described by Cussac et al. (1996). Briefly, membranes (10 μg) from untreated CHO-hD_3 cells and cells preincubated with PTX (100 ng/ml) or cholera toxin (1 μg/ml) for 6 h were incubated for 60 min in buffer containing 8 μM [^{32}P]NAD (2 μCi), 70 mM Tris/HCl, pH 8.0, 1 mM ATP, 0.1 mM GTP, 1 mM EDTA, 25 mM dithiothreitol, 10 mM nicotinamide, 0.1 mM $MgCl_2$, and 100 ng of PTX in a 40-μl assay volume. PTX was preactivated with 25 mM dithiothreitol for 30 min at 37°C. The reaction was stopped by addition of 40 μl of Laemmi buffer 2× and the sample was boiled 3 min at 95°C. Two micrograms of protein from the sample was then separated in 10% polyacrylamide gel and [^{32}P]ADP-ribosylated $G_{i/O}$ proteins were revealed by 8 h exposure of the dried gel to Hyperfilm (Amersham).

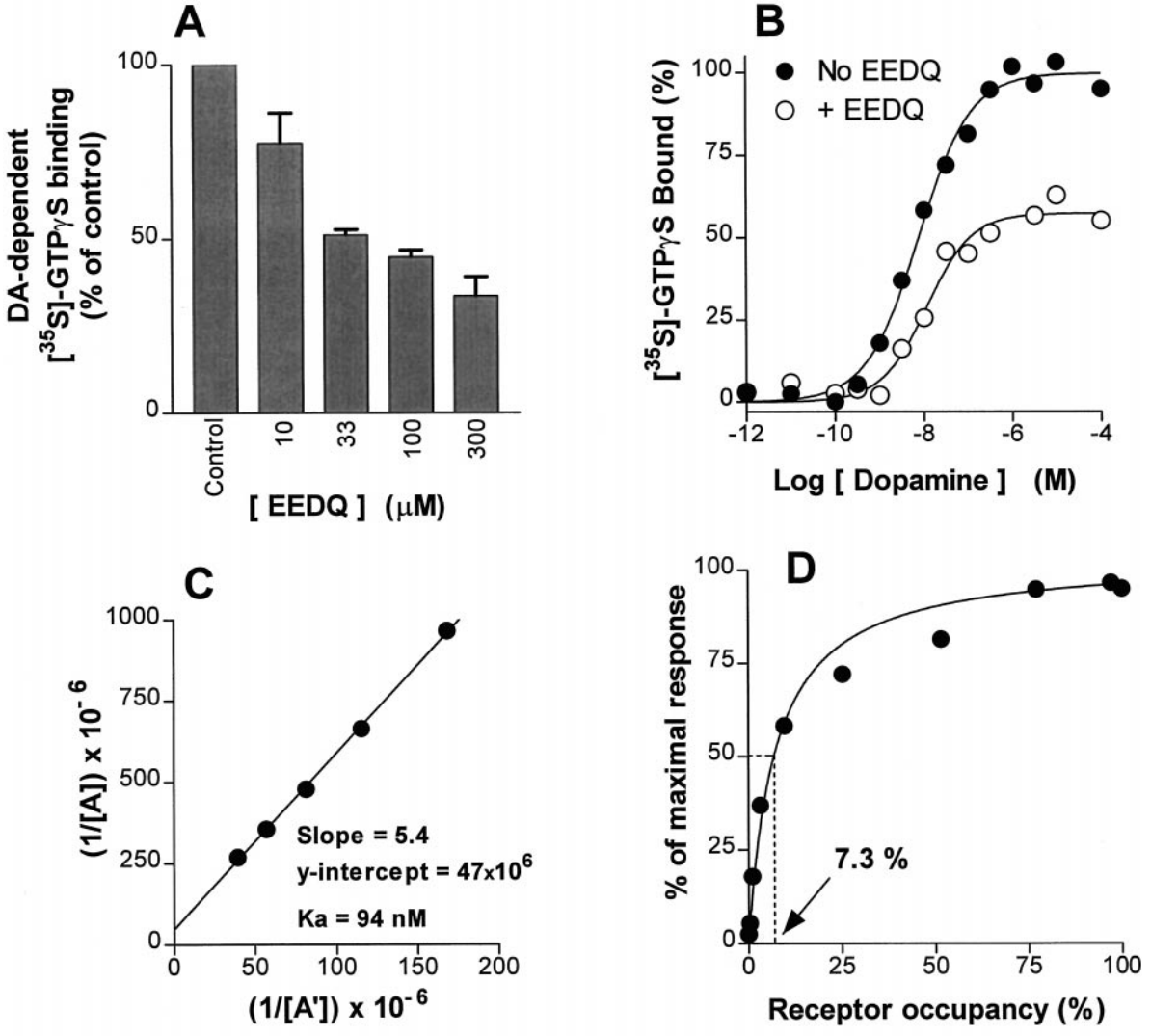

Fig. 3. hD_3 receptor inactivation with EEDQ reveals receptor reserve for dopamine-stimulated [^{35}S]GTPγS binding to CHO-hD_3 membranes. A, concentration-dependent reduction of dopamine-stimulated [^{35}S]GTPγS binding by pretreatment with EEDQ (0 to 300 μM). Columns represent mean ± S.E.M. from at least three experiments carried out in triplicate. B, stimulation by dopamine of [^{35}S]GTPγS binding to control or EEDQ (33 μM)-pretreated CHO-hD_3 membranes. C, double-reciprocal plot of 1/[A] versus 1/[A′], where [A] and [A′] are equiactive concentrations for stimulation of [^{35}S]GTPγS binding with and without EEDQ treatment, respectively. D, dopamine occupancy/response relationship, derived using the value of K_A from C. Hyperbolic isotherm indicates the presence of receptor reserve. For B, C, and D, points shown are means of triplicate determinations from a representative experiment repeated on at least three occasions. Mean K_A value was 53 ± 23 nM. Mean half-maximal response to dopamine was observed at at 11.8 ± 2.3% occupation of hD_3 binding sites.

Antiserum Treatment of CHO-hD_3 or -hD_2 Membranes. CHO-hD_3 and -hD_2 membranes (30–50 μg protein) were preincubated at 4°C for 5 h with 3.3 μg of antisera against different Gα proteins. [^{35}S]GTPγS binding was then performed in absence and in presence of dopamine (10 μM) as described above. The antisera used were the same as described above and were chosen for their capability to recognize the COOH terminal part of Gα subunits involved in receptor interactions. Another antiserum (C17) against c-Jun NH_2-terminal kinase (JNK1), a target unrelated to G proteins, was also tested as a control to exclude nonspecific antibody effects.

Compounds. (+)7-OH-DPAT was obtained from CNRS (Paris, France). PD 128,907 was purchased from RBI (Natick, MA); dopamine, haloperidol, EEDQ, and cholera and PTXs were purchased from Sigma. GR 218,231 and S 14297 were synthesized by J.-L. Peglion, Servier.

Results

Saturation Binding Experiments. The receptor expression level of hD_3 receptors, determined in [^{125}I]iodosulpride saturation binding experiments, was almost 11-fold higher than that of hD_2 receptors (Table 1 and Fig. 1).

The number of dopamine-activated G proteins, determined in [^{35}S]GTPγS isotopic dilution experiments with unlabeled GTPγS, was higher in CHO-hD_3 membranes than in CHO-hD_2 membranes (Table 1). These different expression levels of receptors (R) and G proteins (G) corresponded to a 3-fold higher R/G ratio in CHO-hD_3 membranes than in CHO-hD_2 membranes (4.6:1.5; Table 1). Treatment of CHO-hD_3 membranes with EEDQ (33 μM) reduced hD_3 receptor density by about half (Tables 1 and 2). The effect of EEDQ was specific to the receptors: EEDQ did not significantly alter the number or affinity of [^{35}S]GTPγS for dopamine-activated G proteins (Table 1).

hD_3 Receptor Alkylation with EEDQ. CHO-hD_3 membranes were sensitive to EEDQ treatment. Addition of EEDQ to ice-cold membranes and immediate centrifugation (15 min, 4°C, 20,000*g*) was sufficient to reduce the number of hD_3 binding sites in a concentration-dependent manner without a change in affinity of the radioligand (Table 2 and Fig. 2). EEDQ also concentration dependently reduced the stimulation of [^{35}S]GTPγS binding induced by dopamine (Table 2 and Fig. 3). At the maximal EEDQ concentration tested (300 μM), the density of hD_3 receptors was reduced by over 80%, whereas dopamine-induced [^{35}S]GTPγS binding was reduced by 64%. Subsequent experiments were carried out with an EEDQ concentration of 33 μM, which reduced dopamine-induced [^{35}S]GTPγS binding by about 50% (Tables 1 and 2). Under these conditions, the pEC_{50} ($-\log EC_{50}$) of dopamine was slightly reduced to 7.87 ± 0.09 (compared with 8.00 for control membranes, Table 4), without alteration of basal [^{35}S]GTPγS binding. The K_A value determined by Furchgott analysis was 53 ± 23 nM, which corresponded closely to the affinity of dopamine at hD_3 receptors determined in competition binding experiments (54.9 nM, Table 3). The resulting K_A/EC_{50} ratio for dopamine was 5.3, indicating the presence of receptor reserve. This was confirmed in occupancy/response plots, which yielded hyperbolic curves, with the mean half-maximal response to dopamine being observed at 11.8 ± 2.3% occupation of hD_3 binding sites (Fig. 3).

[^{125}I]Iodosulpride Competition Binding hD_2 and hD_3 At hD_3 receptors, agonist competition binding isotherms were monophasic, although in some experiments with dopamine a small (~10% of binding sites), high-affinity (pK_H, $-\log K_H$, ~ 9) component was apparent (data not shown). At hD_2 receptors, agonist competition isotherms were biphasic and fitted better to a two-site model ($p < .05$, F test; Fig. 4), yielding estimates of affinity for the high- and low-affinity components (Table 3), presumably reflecting binding to G protein-coupled and -uncoupled states of the receptor, respectively. Selectivity ratios of affinity at hD_2/hD_3 receptors were calculated by comparing the K_i at hD_3 receptors with the K_H and the K_L at hD_2 receptors. Competition binding curves with antagonist ligands, haloperidol, S 14297, and GR 218,231, were monophasic for both hD_2 and hD_3 sites (Table 3).

[^{35}S]GTPγS Binding Conditions at CHO-hD_3 and CHO-hD_2 Cell Membranes. In preliminary experiments (not shown), conditions were defined that yielded optimal dopamine-induced stimulation of [^{35}S]GTPγS binding. 1) Op-

TABLE 3

Competition binding of dopaminergic ligands at hD_2 and hD_3 receptors

Affinities (pK_i values) at hD_3 and hD_2 receptors stably expressed in CHO cells were determined by competition binding experiments with [^{125}I]iodosulpride. Two-site analysis of isotherms at hD_2 receptors is shown if this was significantly superior to a one-site fit ($P \leq 0.05$, F test). Percentage of high-affinity binding sites is denoted *% high*. Isotherms at hD_3 receptors fitted best to a single binding site model. Results are means ± S.E. of mean of at least four independent experiments. $K_{i/H/L}$ values were calculated from respective mean $pK_{i/H/L}$ values. hD_2/hD_3 affinity ratio was obtained by dividing $K_{i/H/L}$ value at hD_2 receptors by K_i value at hD_3: for agonist ligands ratios show a wide variation depending on whether K_i value at hD_3 is compared with K_H or K_L value at hD_2.

Ligand	hD_2			hD_3			K_i Ratio hD_2/hD_3
	$pK_{i/H/L}$	$K_{i/H/L}$	nH	pK_i	K_i	nH	
		nM	*% high*		*nM*		
Dopamine							
pK_i				7.26 ± 0.13	54.9	0.83 ± 0.08	
pK_H	7.36 ± 0.08	43.6	0.58 ± 0.02				0.79
pK_L	5.37 ± 0.05	4265	(33.3 ± 2.1)				78
(+)-7-OH-DPAT							
pK_i				8.52 ± 0.04	3.02	0.96 ± 0.04	
pK_H	8.45 ± 0.11	3.54	0.74 ± 0.01				1.2
pK_L	6.93 ± 0.03	117	(20.3 ± 0.5)				39
PD 128,907							
pK_i				8.67 ± 0.11	2.13	0.81 ± 0.03	
pK_H	8.13 ± 0.07	7.41	0.62 ± 0.01				3.5
pK_L	6.07 ± 0.02	851	(22.4 ± 1.0)				400
Haloperidol pK_i	9.37 ± 0.04	0.42	0.91 ± 0.05	8.66 ± 0.05	2.18	0.86 ± 0.05	0.2
S 14297 pK_i	6.48 ± 0.04	331	0.87 ± 0.06	7.91 ± 0.02	12.3	0.90 ± 0.03	27
GR 218,231 pK_i	7.17 ± 0.02	67.6	0.96 ± 0.05	8.95 ± 0.1	1.12	1.03 ± 0.02	60

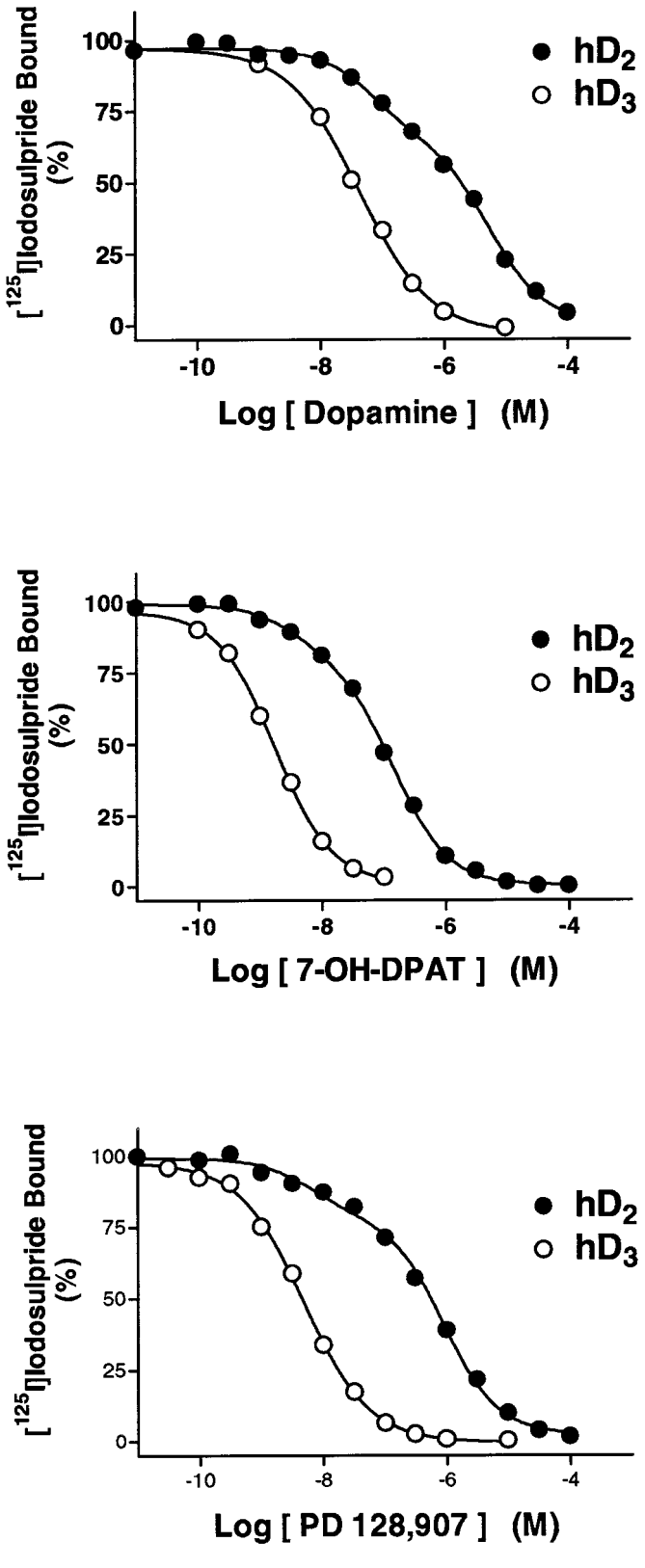

Fig. 4. Competition binding of dopaminergic agonists at hD_3 and hD_2 receptors. Representative [^{125}I]iodosulpride competition binding isotherms for dopamine, (+)-7-OH-DPAT and PD 128,907 at hD_2 and at hD_3 receptors. At hD_2 receptors the data fitted better to a two-site model ($P < .05$, F test). Points shown are means of triplicate determinations from representative experiments repeated on at least three occasions. pK_i, pK_H, and pK_L data from these experiments are shown in Table 3.

timal stimulation was observed at NaCl concentrations of 100 and 150 mM for hD_2 and hD_3 membranes, respectively. 2) GDP concentration dependently reduced basal binding of [^{35}S]GTPγS to both hD_2 and hD_3 cell membranes. 3) $MgCl_2$ increased dopamine-dependent [^{35}S]GTPγS binding to a maximum at around 3 to 10 mM for both receptor subtypes. 4) Stimulation of [^{35}S]GTPγS binding was linear with time over the period of the incubations. In view of the lower stimulation of [^{35}S]GTPγS binding by agonists at hD_3 receptors, a higher concentration of [^{35}S]GTPγS was used (1.0 nM) than with hD_2 (0.1 nM) to provide a stronger signal. Typical binding of [^{35}S]GTPγS (0.1 nM) to CHO-hD_2 membranes was 90 to 100 fmol/mg basal and 230 to 250 fmol/mg in the presence of dopamine (10 μM). Typical binding of [^{35}S]GTPγS (1 nM) to CHO-hD_3 membranes was 1000 to 1100 fmol/mg basal and 1500 to 1600 fmol/mg with dopamine (10 μM). In control experiments in which the concentration of [^{35}S]GTPγS used for hD_3 receptors was 0.1 nM (as for hD_2 receptors), the pEC_{50} for dopamine was 8.04 ± 0.03 (n = 3) nM, not significantly different from the pEC_{50} observed with a [^{35}S]GTPγS concentration of 1 nM (8.00 ± 0.07, Table 4). Dopamine-induced stimulation was 45.8 ± 2.8% (n = 3).

[^{35}S]GTPγS Binding at CHO-hD_3 and CHO-hD_2 Cell Membranes: Agonist Actions. Dopamine, PD 128,907, and (+)-7-OH-DPAT increased [^{35}S]GTPγS binding to CHO-hD_3 and CHO-hD_2 membranes in a concentration-dependent manner, with EC_{50}, E_{max}, and nH values shown in Table 4. S 14297 exhibited slight agonist actions at hD_2 receptors (E_{max} = 20.6%) but no agonist activity was detected at hD_3 receptors (Fig. 5). PD 128,907 was almost twice as efficacious at hD_2 as at hD_3 receptors, whereas (+)-7-OH-DPAT was a partial agonist at both receptor subtypes. The ratios of EC_{50} values at hD_2/hD_3 were intermediate between the $K_H(hD_2)/K_i(hD_3)$ and the $K_L(hD_2)/K_i(hD_3)$ ratios in Table 3. In control experiments in which the concentrations of NaCl and $MgCl_2$ were inverted between hD_2 and hD_3, we did not observe marked changes in EC_{50} and E_{max} values of (+)-7-OH-DPAT and PD 128,907 (data not shown), but a slight decrease in percentage of stimulation by agonists was noted.

[^{35}S]GTPγS Binding at CHO-hD_3 and CHO-hD_2 Cell Membranes: Antagonist Actions. Haloperidol, GR 218,231, and S 14297 did not alter [^{35}S]GTPγS binding from basal levels at hD_3 receptors or, except S 14297 (as described above) at hD_2 receptors. The inhibition of dopamine-stimulated [^{35}S]GTPγS binding (Table 5 and Fig. 6), yielded antagonist potencies (K_B values), which conserved the same order of potency as the K_i values shown for these compounds in Table 3. The novel ligand GR 218,231 was shown to behave as a competitive antagonist, inducing a rightward parallel shift of the dopamine stimulation curve without loss of maximal efficacy (Fig. 7), yielding a linear Schild plot (r = 0.96, slope = 1.06 ± 0.10) and a pA_2 value of 9.34 similar to its affinity calculated by competition binding (pK_i = 8.95, Table 3).

Effect of Pertussis and Cholera Toxins on hD_3 Receptor Coupling. Membranes were prepared from CHO-hD_3 cells treated with PTX (100 ng/ml) or cholera toxin (1 μg/ml) for 6 h. Pretreatment with PTX reduced, but did not totally suppress, dopamine-dependent [^{35}S]GTPγS binding: it was attenuated by about 80% (81 ± 16 fmol/mg versus 430 ± 26 fmol/mg in control), without changes in basal [^{35}S]GTPγS binding (Fig. 8). The incomplete suppression of dopamine-stimulated [^{35}S]GTPγS binding was not due to an

insufficiently long incubation of CHO-hD_3 cells with PTX. Indeed, when membranes were prepared from CHO-hD_3 cells after the 6-h incubation, no subsequent incorporation of [^{32}P]ADP-ribose was observed, indicating that all the $G\alpha_{i/o}$ proteins present had already been ADP-ribosylated (Fig. 8).

Dopamine stimulated [^{35}S]GTPγS binding to membranes of CHO-hD_3 cells treated with cholera toxin with an pEC_{50} of 8.04 ± 0.08 (n = 4), similar to that observed in control membranes (pEC_{50} = 8.00, Table 4), but basal [^{35}S]GTPγS binding in cholera toxin-treated cell membranes was increased (1230 ± 80 fmol/mg versus 1030 ± 36 fmol/mg for control membranes). However, the amount of dopamine-dependent [^{35}S]GTPγS binding was unchanged (407 ± 61 fmol/mg versus 430 ± 26 fmol/mg in control membranes) (Fig. 8).

Effect of Antibodies on hD_3 and hD_2 Receptor Coupling. The presence of $G\alpha_{i/o}$, $G\alpha_S$, and $G\alpha_{q/11}$ in both CHO-hD_3 and CHO-hD_2 cell membranes was demonstrated by immunodetection with specific antibodies (Fig. 9). Preincubation of hD3 and hD_2 cell membranes with anti-$G\alpha_i/\alpha_o$ subunit antiserum significantly ($P < .05$, Student's paired t test) attenuated dopamine-dependent [^{35}S]GTPγS binding, at both hD_3 and hD_2 receptors (Fig. 9). Anti-$G\alpha_q/\alpha_{11}$ antiserum significantly attenuated dopamine-dependent [^{35}S]GTPγS binding to CHO-hD_3 but not CHO-hD_2 membranes ($P < .05$, Student's paired t test). Antisera directed against $G\alpha_S$ and an unrelated target, JNK1, did not affect [^{35}S]GTPγS binding at either receptor (data not shown).

Discussion

The primary purpose of the present study was to investigate the G protein coupling of dopamine hD_3 receptors. The results demonstrate that hD_3 (and hD_2) receptors mediate stimulation of [^{35}S]GTPγS binding when expressed in mammalian CHO cells, indicating that they are capable of activating intracellular G proteins. A robust degree of stimulation was observed (Fig. 5), enabling a detailed investigation of the coupling of these receptor subtypes and the identification of some marked differences between hD_3 and hD_2 sites.

First, despite the 11-fold higher hD_3 receptor expression level (15 pmol/mg), the dopamine-elicited increase in [^{35}S]GTPγS binding (up to 1.6-fold) was less than that at hD_2 receptors. Partial inactivation of hD_3 receptors using the alkylating agent EEDQ showed that high hD_3 receptor expression levels are necessary for stimulation of G protein activation, because EEDQ treatment reduced the stimulation of [^{35}S]GTPγS binding induced by dopamine (Table 2). Nevertheless, Furchgott analysis yielded a hyperbolic occupancy/response plot (Fig. 3), indicating the presence of marked receptor reserve for half-maximal stimulation of [^{35}S]GTPγS binding by dopamine and is consistent with the 5-fold K_A/EC_{50} ratio for activation of hD_3 receptors (see *Results*). Thus the limited stimulation of [^{35}S]GTPγS binding to CHO-hD_3 membranes appears to be a property of hD_3 receptors themselves and not due to insufficient intrinsic efficacy of dopamine. It should be noted that the modest stimulation at hD_3 receptors is not a consequence of augmented basal [^{35}S]GTPγS binding, because basal [^{35}S]GTPγS binding was unaffected by receptor inactivation (not shown). Furthermore, the low-fold stimulation in CHO-hD_3 membranes is unlikely to be due to a global lack of activatable G proteins: the amount (B_{max}) of dopamine-activated G proteins in CHO-hD_3 cell membranes is about 3-fold higher than that in CHO-hD_2 cell membranes (Table 1 and Fig. 1). Taken together, the present data suggest that stimulation of hD_3 receptors less effectively induces the conformational changes necessary for G protein activation than at hD_2 receptors (Chio et al., 1994), perhaps due to a slower rate of G protein coupling/uncoupling at hD_3 receptors or, alternatively, to interaction with different G protein subtypes at hD_3 versus hD_2 receptors, a possibility discussed below.

Second, agonist efficacy varied between hD_2 and hD_3 receptors. S 14297, previously characterized as a D_3 receptor antagonist in vivo (Millan et al., 1995a,b) exhibited residual intrinsic activity at hD_2 receptors (Table 4) but no detectable agonist activity at hD_3 receptors. In the present high-expressing CHO-hD_3 cell membranes, it might have been expected that partial agonist actions at hD_3 receptors would be "amplified" to yield maximal activation of [^{35}S]GTPγS binding, like dopamine. Nevertheless, both (+)-7-OH-DPAT and PD 128,907 behaved as partial agonists at hD_3 receptors (Table 4 and Fig. 5). It therefore appears that despite the high levels of hD_3 receptors, the high R/G ratio in CHO-hD_3 membranes, and, as discussed above, receptor reserve for activation by dopamine, the present [^{35}S]GTPγS binding methodology more readily differentiates partial agonist efficacies at hD_3 receptors than certain downstream models of hD_3 (or hD_2) receptor activation, such as mitogenesis (Sautel et al., 1995), where dopamine, (+)-7-OH-DPAT and PD 128,907 all behaved as full agonists. Furthermore, in the present study, the degree of selectivity of (+)-7-OH-DPAT and PD 128,907 for activation of hD_3 versus hD_2 receptors was greater than previously reported (Levant, 1997). (+)-7-OH-DPAT displayed an hD_3/hD_2 EC_{50} ratio of 77 (Table 4) compared with 14 and 7 for inhibition of adenylyl cyclase and mitogenesis experiments respectively (Chio et al., 1994; Sau-

TABLE 4

Stimulation of [^{35}S]GTPγS binding by dopaminergic ligands at hD_2 and hD_3 receptors

Agonist efficacies were determined by [^{35}S]GTPγS binding. Results are means ± S.E. of mean of at least three independent experiments. EC_{50} values were calculated from mean pEC_{50} values. Haloperidol and GR 218,231 did not induce any alteration of [^{35}S]GTPγS binding to either hD_2 or hD_3 membranes. S 14297 did not alter [^{35}S]GTPγS binding to hD_3 membranes.

Agonist	hD_2				hD_3				EC_{50} Ratio hD_2/hD_3
	pEC_{50}	EC_{50}	E_{MAX}	nH	pEC_{50}	EC_{50}	E_{MAX}	nH	
		nM	%			*nM*	%		
Dopamine	6.45 ± 0.03	350	103.5 ± 4.9	1.10 ± 0.10	8.00 ± 0.07	10	100.1 ± 3.2	0.82 ± 0.07	35
(+)-7-OH-DPAT	7.35 ± 0.11	44.6	64.3 ± 2.8	0.86 ± 0.10	9.24 ± 0.26	0.58	55.4 ± 4.2	1.27 ± 0.24	77
PD 128,907	6.33 ± 0.10	467	116 ± 3.8	0.73 ± 0.18	8.91 ± 0.12	1.22	63.6 ± 2.8	1.21 ± 0.31	382
S 14297	7.31 ± 0.08	48.6	20.6 ± 1.9	1.53 ± 1.11	n.c.	n.c.	0	n.c.	n.c.

n.c., not computable.

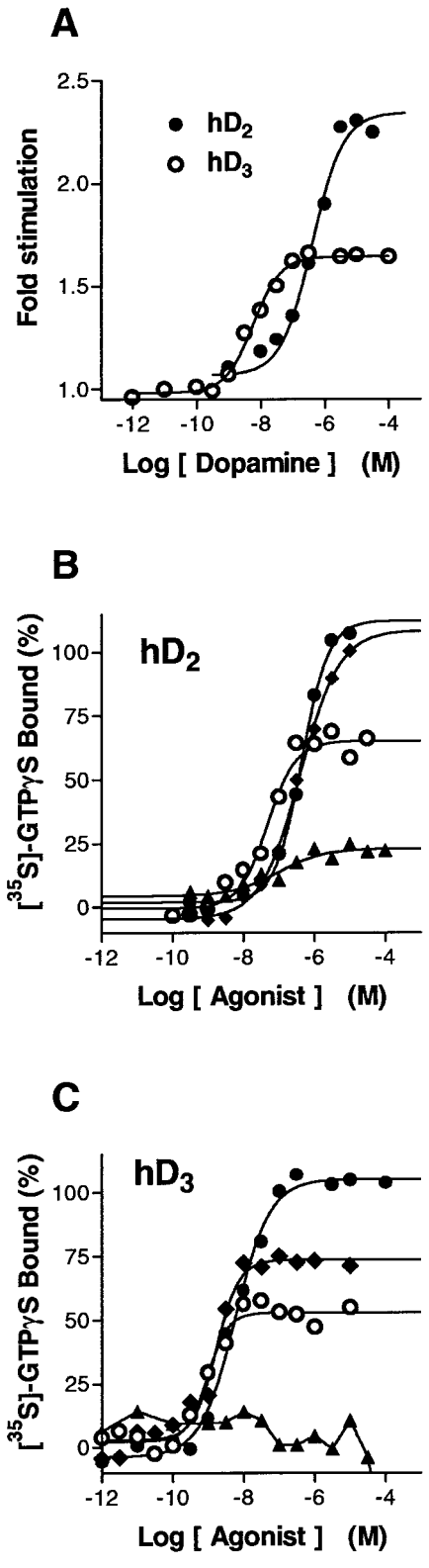

Fig. 5. Agonist stimulation of hD_3 and hD_2 receptor-mediated G protein activation. [^{35}S]GTPγS binding is expressed as a percentage of maximal stimulation given by dopamine. A, fold stimulation of [^{35}S]GTPγS binding by dopamine at hD_2 and hD_3 receptors. B, agonist concentration-response curves at hD_2 receptors. C, agonist concentration-response curves at hD_3 receptors (● dopamine; ♦ PD 128,907; ○ (+)-7-OH-DPAT; and ▲ S 14297). Points shown are means of triplicate determinations from representative experiments repeated on at least three occasions. E_{max}, pEC_{50}, and nH data from these experiments are shown in Table 4.

tel et al., 1995). PD 128,907 was 382-fold as selective in this study compared with only 6-fold as selective in mitogenesis experiments (Pugsley et al., 1995). The source of these differences is unclear but probably relates to the fact that mitogenesis and extracellular acidification measure responses that are distal to agonist-induced receptor/G protein conformational changes. In contrast, [^{35}S]GTPγS binding measures G protein activation, which provides a more proximal indication of agonist/antagonist actions at the receptor itself.

Third, the antagonist rank order of potency of haloperidol, S 14297, and the novel selective antagonist, GR 218,231 (Murray et al., 1996; Figs. 6 and 7) at hD_3 and hD_2 receptors corresponded to the order of affinity determined in competition binding experiments, although a reduced preference for hD_3 sites was observed in functional tests (Table 5). It is noteworthy that, whereas the pK_B values of the antagonists resembled their respective pK_i values, the antagonists did not exhibit negative efficacy at either hD_3 or hD_2 receptors at concentrations up to 10^{-5} M. At higher concentrations, [^{35}S]GTPγS binding was somewhat reduced below basal levels in some experiments but this was taken to be a nonspecific effect, because it occurred at concentrations >1000-fold greater than their binding affinity, the effects did not show a discernible correlation with the order of potency, and a similar trend was observed in untransfected CHO cell membranes (D. Cussac, unpublished observations). A recent study using hD_3 receptors expressed in CHO cells (Malmberg et al., 1998) reported that basal [^{35}S]GTPγS binding could be increased by dopaminergic agonists and decreased by antagonists. However, in that study dopamine-induced stimulation was very low (only ~1.2-fold), negative efficacy was only observed at very high drug concentrations ($\geq 10^{-6}$ M), and control untransfected CHO cells were not examined. Nevertheless, further investigation of the issue of negative efficacy is desirable, because the conditions used for [^{35}S]GTPγS binding both in the present study and that of Malmberg et al. (1998) (high concentrations of GDP and NaCl) favor suppression of constitutive hD_3 and hD_2 receptor activation (Gardner et al., 1996). Indeed, some studies reported that haloperidol shows negative efficacy in models of D_3 and D_2 receptor activation (mitogenesis, Griffon et al., 1996; prolactin secretion, Nilsson et al., 1996).

Fourth, G protein activation by dopamine at hD_3 receptors is PTX sensitive (Fig. 8), implicating $G_{i/o}$ G proteins. This is analogous to the known PTX sensitivity of D_2 receptors (Neve et al., 1989; Lajiness et al., 1993; Seabrook et al., 1994; Swarzenski et al., 1996; Hall and Strange, 1997). However, marked differences were observed between hD_3 and hD_2 receptors in antibody tests. In the present study, dopamine-

TABLE 5

Antagonism of dopamine-stimulated [^{35}S]GTPγS binding to CHO-hD_2 and -hD_3 membranes

Antagonist potencies (pK_B values) were calculated from IC_{50} values for inhibition of dopamine (3 μM for hD_2, 1 μM for hD_3)-stimulated [^{35}S]GTPγS binding. pK_B values are expressed as means ± S.E. of mean of at least three independent experiments. K_B values were calculated from mean pK_B values.

Antagonist	hD_2		hD_3		K_B Ratio hD_2/hD_3
	pK_B	K_B	pK_B	K_B	
		nM		*nM*	
Haloperidol	9.30 ± 0.13	0.50	7.67 ± 0.27	21.3	0.02
S 14297	6.56 ± 0.09	275	7.53 ± 0.08	29.5	9.3
GR 218231	7.17 ± 0.16	67.6	8.65 ± 0.03	2.23	30

stimulated [^{35}S]GTPγS binding at hD_3 and hD_2 receptors was inhibited by an antiserum that recognizes the three α_i subunits ($\alpha_{i1/i2/i3}$) and, more weakly, α_O subunits (Cussac et al., 1996). This antiserum inhibited dopamine-stimulated [^{35}S]GTPγS binding to CHO-hD_3 membranes more strongly than to CHO-hD_2 membranes (67% versus 40% inhibition, Fig. 9). The greater effect at hD_3 receptors may be due to a coupling by hD_3 to both G_i and G_O proteins, whereas hD_2 receptors may couple only to members of the G_i protein family. This would be consistent with a study that found that an attenuation of D_2 receptor-mediated inhibition of adenylyl cyclase activity was achieved by pretreatment with anti-$G\alpha_{i1/i2}$ but not by anti-$G\alpha_O$ antibodies (Izenwasser and Côté, 1995). Alternatively, hD_3 receptor functional coupling to G proteins may be more labile than that at hD_2 receptors. Thus, the hD_3 receptor/G protein interaction may be more susceptible to the steric hindrance of antibody binding to Gα subunits, in accordance with the apparently less "efficient" G protein coupling of hD_3 receptors discussed above.

Fifth, an interaction of hD_3 receptors with a G protein other than G_i or G_O is suggested by the observation that, when CHO-hD_3 cells were treated with PTX, there remained a residual capacity of dopamine to stimulate [^{35}S]GTPγS binding. This was not due to an insufficient incubation period with PTX, because control experiments (Fig. 8) indicated that 6 h were sufficient to completely ADP-ribosylate $G_{i/o}$ proteins in CHO-hD_3 cells, in agreement with previous studies in pituitary cells (Cussac et al., 1996). Thus, a component of dopamine-dependent [^{35}S]GTPγS binding at hD_3 receptors may be mediated by a G protein that is not PTX sensitive. This possibility is supported by the inhibition of dopamine-dependent [^{35}S]GTPγS binding at hD_3 receptors by anti-$G\alpha_{q/11}$ antibodies (Fig. 9). Such an effect was not observed at hD_2 receptors, although $\alpha_{q/11}$ (as well as $\alpha_{i/O}$ and α_S) subunits are expressed in both cell lines (Fig. 9). Furthermore, the inhibition by anti-$G\alpha_{q/11}$ antibodies is not simply due to nonspecific Ig interactions, because the same concentration of antisera against an unrelated target (JNK1) did not affect dopamine-dependent [^{35}S]GTPγS binding (not shown). Thus, these data suggest that hD_3 receptors in the present CHO-hD_3 cell line may interact with $G\alpha_{q/11}$ and, potentially, modulate phosphatidyl inositol turnover. Although a previous study of hD_3 receptors expressed in CHO cells did not find such an effect (Freedman et al., 1994), that may have been due to a 50-fold lower hD_3 expression level (0.3 versus 15 pmol/mg in the cells used in this study).

In conclusion, the [^{35}S]GTPγS binding strategy employed in this study enabled the characterization of G protein coupling at hD_3 receptors. The data suggest that the coupling of hD_3 receptors to G proteins is less efficacious than that at hD_2 receptors, yielding a less pronounced stimulation of [^{35}S]GTPγS binding despite the high expression levels of receptors and G proteins, and the presence of receptor reserve for dopamine. In addition, unlike hD_2 receptors, hD_3 receptors may couple to G proteins other than G_i, such as G_O and/or $G_{q/11}$ proteins. The precise G protein subtypes in-

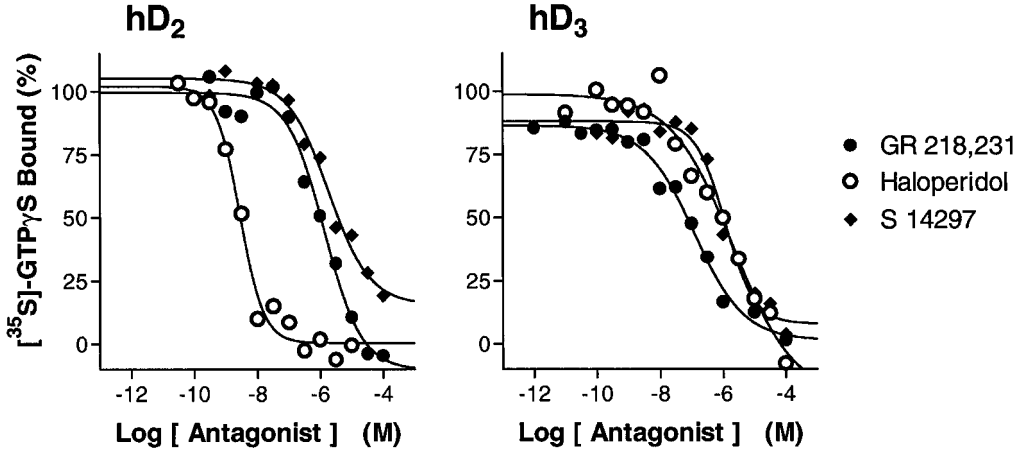

Fig. 6. Antagonism of hD_3 and hD_2 receptor-mediated G protein activation. Antagonism of dopamine (3 μM)-stimulated [^{35}S]GTPγS binding at hD_2 receptors and of dopamine (1 μM)-stimulated [^{35}S]GTPγS binding at hD_3 receptors (○ haloperidol, ● GR 218,231, and ♦ S 14297). Points shown are means of triplicate determinations from representative experiments repeated on at least three occasions. pIC_{50} and pK_B data from these experiments are shown in Table 4.

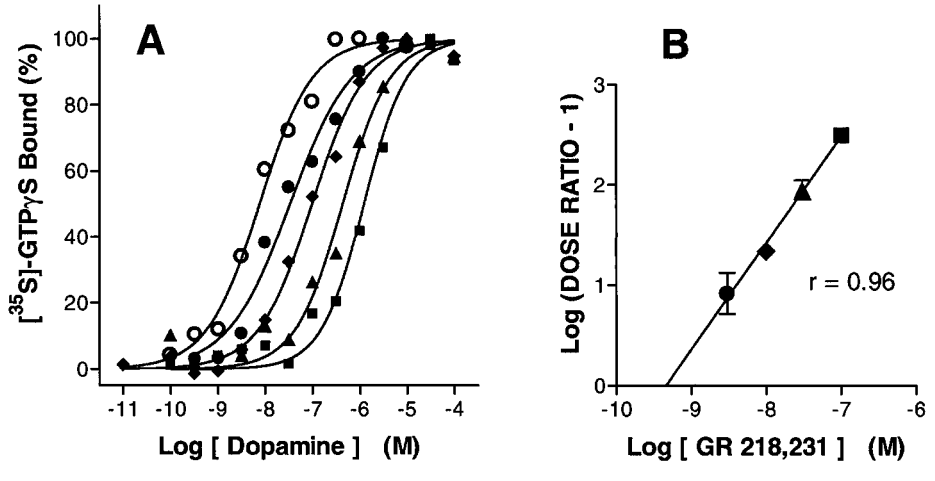

Fig. 7. Competitive antagonism of hD_3 receptor-mediated G protein activation by GR 218,231. A, concentration-response isotherms for stimulation of [^{35}S]GTPγS binding by dopamine at hD_3 receptors in the presence of increasing concentrations of GR 218,231 (● 3 nM, ♦ 10 nM, ▲ 30 nM, and ■ 100 nM). Points shown are means of triplicate determinations from representative experiments repeated on at least three occasions. B, Schild plot of the hD_3 dopamine concentration-response experiments. Points shown are mean values from at least three independent experiments performed in triplicate.

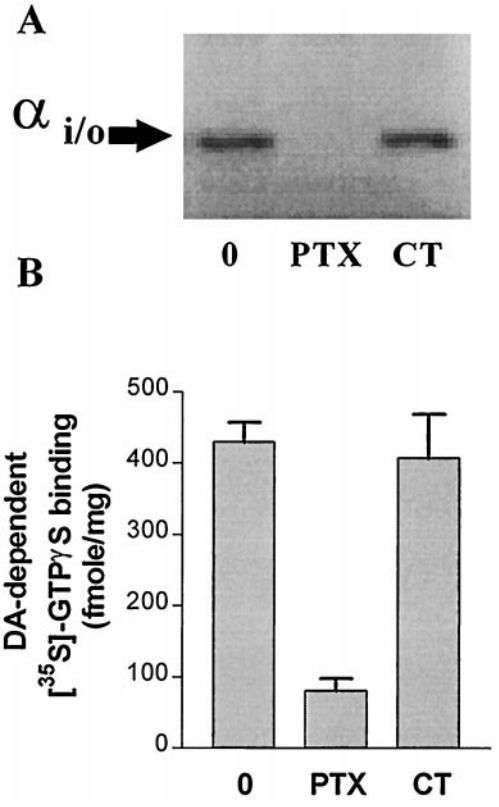

Fig. 8. Partial attenuation of dopamine-stimulated [^{35}S]GTPγS binding by PTX, but not cholera toxin. A, [^{32}P]ADP-ribose incorporation catalyzed by PTX. ADP-ribosylation of CHO-hD_3 cell membranes preincubated with or without PTX or cholera toxin (CT) for 6 h was carried out as described in *Materials and Methods.* [^{32}P]ADP-ribosylated $G_{i/O}$ proteins were revealed by a 8-h exposure to Hyperfilm. The data shows that PTX pretreatment abolished subsequent [^{32}P]ADP-ribose incorporation. The same result was obtained in a second, independent experiment. B, Effect of PTX and CT on dopamine-stimulated [^{35}S]GTPγS binding. CHO-hD_3 cell were incubated for 6 h with PTX or CT and stimulation of [^{35}S]GTPγS binding was determined with dopamine (10 μM). Bars represent mean ± S.E.M. values from at least three independent experiments performed in triplicate and are expressed in fentomoles per milligram of dopamine-induced [^{35}S]GTPγS binding.

volved in hD_3 receptor coupling and their relevance to physiological actions require further investigation.

Acknowledgment

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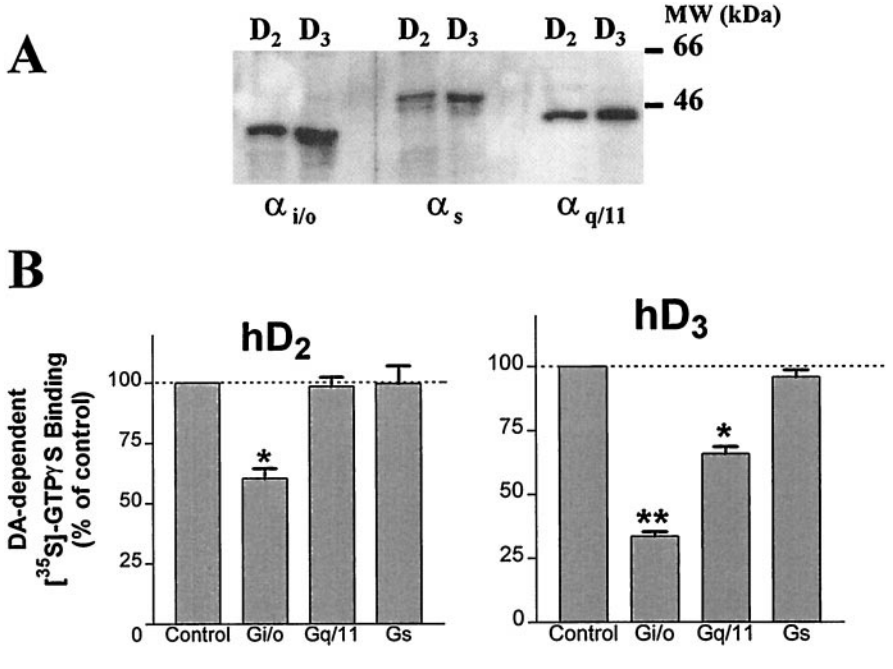

Fig. 9. Inhibition by anti-G protein antibodies of dopamine-stimulated [^{35}S]GTPγS binding to CHO-hD_3 and CHO-hD_2 cell membranes. A, immunodetection of $G\alpha_{i/o}$, $G\alpha_S$, and $G\alpha_{q/11}$ subunits in both CHO-hD_3 and CHO-hD_2 cell membranes was performed as described in *Materials and Methods*. B, CHO-hD_2 and CHO-hD_3 cell membranes were incubated for 5 h at 4°C in the presence of antibodies used in A. Stimulation of [^{35}S]GTPγS binding was determined with dopamine (10 μM). Bars represent mean ± S.E.M. values from at least three independent experiments performed in triplicate and are expressed as percentage of dopamine-dependent [^{35}S]GTPγS binding observed in control (untreated) samples. $^*P < .05$; $^{**}P < .01$ versus control (2-tailed, paired t test).

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