G Protein Activation by Human Dopamine D3 Receptors in High-Expressing Chinese Hamster Ovary Cells: A Guanosine-5’-O-(3-[35S]thio)-Triphosphate Binding and Antibody Study

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ABSTRACT

Despite extensive study, the G protein coupling of dopamine D3 receptors is poorly understood. In this study, we used guanosine-5’-O-(3-[35S]thio)-triphosphate ([35S]-GTPγS) binding to investigate the activation of G proteins coupled to human (h) D3 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 hD3 receptor occupation with the alkylating agent, EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline). Thus, half-maximal stimulation of [35S]-GTPγS binding required only 11.8% receptor occupation of hD3 sites. In contrast, although the hD2short 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 hD3 and, less potently, at hD2 receptors by the preferential D3 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-([dipropylamino]-6-(4-methoxyphenylsulfonyl)methyl)-1,2,3,4-tetrahydronaphtalene), blocked dopamine-stimulated [35S]-GTPγS binding at hD2 receptors, attenuated hD2-induced G protein activation at hD3 receptors, and 3) hD3 receptors may couple to different G protein subtypes than hD2 receptors, including non-pertussis sensitive Gα11 proteins.

Dopaminergic neurotransmission is mediated by five receptor subtypes (D1 to D5) which can be grouped into two receptor families. D1-like receptors include the D1 and D5 subtypes, whereas D2-like receptors include the D2, D3, and D4 subtypes. D2 and D3 receptors, in particular, display marked sequence homology and pharmacological similarity in their in vitro ligand binding profiles (Levant, 1997; Missale et al., 1998). However, D3 receptors may be distinguished from D2 receptors by several factors. D3 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 D2 receptors couple efficiently to second-messenger systems, markedly inhibiting adenylyl cyclase activity, such responses have proved elusive and complex for D3 receptors (e.g., Freedman et al., 1994; MacKenzie et al., 1994; Tang et al., 1994; Griffon et al., 1997). Indeed, D3 receptors couple selectively to inhibition of adenylyl cyclase type V, but not type I or VI, and only weakly to type II (Robinson and

ABBREVIATIONS: EEDQ, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; GR 218,231, 2(R,S)-(dipropylamino)-6-(4-methoxyphenylsulfonylmethyl)-1,2,3,4-tetrahydronaphtalene; [35S]GTPγS, guanosine-5’-O-(3-[35S]thio)-triphosphate; (+/-(dipropylamino)-6-(4-methoxyphenylsulfonylmethyl)-1,2,3,4-tetrahydronaphtalene; (+)-7-OH-DPAT, 7-hydroxy-2-(di-n-propylamino)tetralin).
Caron, 1997; Watts and Neve, 1997). In vitro studies of agonist efficacy have employed other measures of receptor activation, including medium acidification (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 neurons is unclear. A more promising approach may be to measure receptor-mediated G protein activation by stimulation of guanosine-5′-O-(3-thio)-triphosphate ([35S]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) D3 receptors. In addition, in some tests results at hD3 receptors were compared with those at hD2 receptors. First, differences in the second-messenger actions of D3 and D2 receptors may be related to differing capacities for stimulation of G proteins. We addressed this issue by investigating the ability of hD3 receptors to mediate dopamine-stimulated [35S]GTPγS binding. Second, the relationship between binding affinity and functional potency of dopaminergic agonists and antagonists was investigated using the most potent and selective D3 receptor ligands reported to date: the agonists (+)-7-OH-DPAT (7-hydroxy-2-(di-n-propylamino)tetrinal) and PD 128,907 (1,4-(4aR,10B)-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(2,3-b)/dihydro-2-furane) and GR 218,231 (2(R,S)-(dipropylamino)-6-(4-methoxyphenylsulfonylmethyl)-1,2,3,4-tetrahydronaphthalene) (Millan et al., 1995b; Murray et al., 1996). The hD3/hD2 selectivities based on Kᵣ ratios were compared with those based on EC5₀ and Kᵢ Ratios (Burris et al., 1995; Levant, 1997). Third, the signal transduction differences between D3 and D2 receptors, such as the differential coupling to adenyl 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: G₁₅, G₁₇, G₁₁, and G₁₂/₁₃ (Simon et al., 1991). Although a previous study suggested differences in coupling profiles of D2 and D3 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 D₃ 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 [35S]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).

### Materials and Methods

#### Membrane Preparations of Chinese Hamster Ovary (CHO)-hD3 and CHO-hD2 Cells.

CHO cells expressing hD3 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 MgCl₂. The suspension was then centrifuged at 20,000g for 15 min at 4°C and the pellet was resuspended in the appropriate binding buffer (see below) and stored.

### Table 1

Densities of recombinant receptors and agonist-activated G proteins in CHO cells stably expressing hD3 and hD2 receptors

<table>
<thead>
<tr>
<th>Receptor Saturation</th>
<th>CHO-hD3</th>
<th>CHO-hD2 (EEDQ)</th>
<th>CHO-hD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bₘₐₓ (pmol/mg)</td>
<td>15.43 ± 1.33</td>
<td>7.36 ± 1.17</td>
<td>3.38 ± 0.38</td>
</tr>
<tr>
<td>Kᵣ (nM)</td>
<td>1.18 ± 0.19</td>
<td>1.31 ± 0.03</td>
<td>1.68 ± 0.30</td>
</tr>
<tr>
<td>G Protein Saturation</td>
<td>3.38 ± 0.51</td>
<td>2.58 ± 0.53</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>Bₘₐₓ (pmol/mg)</td>
<td>6.72 ± 1.29</td>
<td>6.18 ± 1.08</td>
<td>1.33 ± 0.92</td>
</tr>
<tr>
<td>Kᵣ (nM)</td>
<td>4.6</td>
<td>2.9</td>
<td>1.08</td>
</tr>
</tbody>
</table>

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

### Figure 1

**A** Saturation binding of [35S]iodosulpride and [35S]GTPγS to CHO-hD3 and CHO-hD2 cell membranes. A, representative saturation binding isotherms of [35S]iodosulpride to CHO-hD2 and CHO-hD3 membranes. Basal and dopamine (10 μM)-stimulated [35S]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 [35S]GTPγS binding. Points show means of duplicate determinations from representative experiments repeated on at least four occasions. Bₘₐₓ and Kᵣ/apparent Kᵣ/ are data from these experiments shown in Table 1.
at -80°C. CHO-hD2(short) cell membranes were purchased from Receptor Biology (Baltimore, MD). The “short” hD2 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).

**Table 2**

Concentration-dependent actions of EEDQ on receptor density and function in CHO-hD3 membranes

CHO-hD3 cell membranes were treated with different concentrations of EEDQ. Receptor density was determined in [125I]iodosulpride saturation binding experiments. Percentage of remaining hD3 binding sites is calculated as a percentage of IC50 values, where IC50 is the inhibitory concentration 50 of the antagonist. Percentage of remaining hD3 binding sites is calculated as a percentage of IC50 values according to the Cheng-Prussoff equation. The goodness of fit was tested by runs test. For competition binding experiments, membranes were incubated with [125I]iodosulpride (0.1 mM for hD2 and 0.2 mM for hD3) 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 MgCl2. 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 IC50 values. Inhibition constants (Ki values) were derived from IC50 values according to the Cheng-Prussoff equation. The goodness of fit was tested by runs test. For competition binding experiments, membranes were carried out one- and two-site fits by comparison with a control.

**Figure 2.** Concentration-dependent reduction of hD3 receptor density by EEDQ. A, representative saturation binding isotherms of [125I]iodosulpride to CHO-hD3 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. Bmax and Ki, data from these experiments are shown in Table 2.

by dopamine at hD3 and hD2 receptors was determined by measuring the stimulation of [35S]GTPγS (1332 Ci/mmol; NEN, Les Ulis, France) binding induced by dopamine. CHO-hD3 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 MgCl2, 150 mM NaCl, and 0.1 nM [35S]GTPγS. CHO-hD3 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 MgCl2, 100 mM NaCl, and 1.0 nM [35S]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 [35S]GTPγS. Ki values for inhibition of dopamine (1 and 3 μM for hD3 and hD2 respectively)-stimulated [35S]GTPγS binding were calculated according to Lazareno and Birdsall (1993):

\[
K_i = \frac{IC_{50}}{\left(2 + (agonist/EC_{50})^{nH}\right)} - 1;
\]

where IC50 is the inhibitory concentration of the antagonist, agonist is the dopamine concentration, EC50 is the effective concentration 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, pA2 values were derived by Schild analysis. In isotopic dilution experiments, the basal and dopamine (10 μM)-stimulated binding of radio-labeled [35S]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 [125I]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 at least 3 independent determinations. Protein concentration was determined colorimetrically using a bicinchonic acid assay kit (Sigma Chemical Co., St. Quentin Fallavier, France).

**Table 2**

Concentration-dependent actions of EEDQ on receptor density and function in CHO-hD3 membranes

CHO-hD3 cell membranes were treated with different concentrations of EEDQ. Receptor density was determined in [125I]iodosulpride saturation binding experiments. Percentage of remaining hD3 binding sites is calculated as a percentage of IC50 values, where IC50 is the inhibitory concentration 50 of the antagonist. Percentage of remaining hD3 binding sites is calculated as a percentage of IC50 values according to the Cheng-Prussoff equation. The goodness of fit was tested by runs test. For competition binding experiments, membranes were carried out one- and two-site fits by comparison with a control.
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]GTP\(_\gamma\)S 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 \(\mu\)M EEDQ. Plots were derived of 1/[A] versus 1/[A']; where [A] and [A'] are equiactive concentrations for stimulation of [\(^{35}\)S]GTP\(_\gamma\)S binding before and after receptor alkylation, respectively. \(K_A\) was calculated from

\[ K_A = \frac{(\text{slope})-1}{y\text{-intercept}}. \]

Percentage receptor occupancy \((O)\) was calculated by

\[ O = 100 \times \frac{L}{L + K_A}; \]

where \(L\) is the concentration of agonist.

**Characterization of G proteins by Immunoblotting and ADP-Ribosylation.** Immunoblotting of Go subunits was performed using antisera purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) raised against Go\(_{i/o}\) (C10), Go\(_{\alpha}\) (C18), and Go\(_{q/11}\) (C19). Approximately 2 \(\mu\)g 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 \(\mu\)g) from untreated CHO-hD\(_3\) cells and cells preincubated with PTX (100 ng/ml) or cholera toxin (1 \(\mu\)g/ml) for 6 h were incubated for 60 min in buffer containing 8 \(\mu\)M \(^{32}\)P NAD (2 \(\mu\)Ci), 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-\(\mu\)l assay volume. PTX was preactivated with 25 mM dithiothreitol for 30 min at 37°C. The reaction was stopped by addition of 40 \(\mu\)l of Laemmli 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 Go\(_{i/o}\) 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]GTP\(_\gamma\)S binding to CHO-hD\(_3\) membranes. A, concentration-dependent reduction of dopamine-stimulated [\(^{35}\)S]GTP\(_\gamma\)S binding by pretreatment with EEDQ (0 to 300 \(\mu\)M). Columns represent mean ± S.E.M. from at least three experiments carried out in triplicate. B, stimulation by dopamine of [\(^{35}\)S]GTP\(_\gamma\)S binding to control or EEDQ (33 \(\mu\)M)-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]GTP\(_\gamma\)S 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 ± 2.9% 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 antiserum 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 unlabelled 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 K variation depending on whether hD 2 receptors is shown if this was significantly superior to a one-site fit (Table 1).

[125]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$_i$ and the K$_i$ 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).

TABLE 3

<table>
<thead>
<tr>
<th>Ligand</th>
<th>hD$_3$</th>
<th>hD$_2$</th>
<th>K Ratio hD$_3$/hD$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK$_i$</td>
<td>K$_i$</td>
<td>nH</td>
</tr>
<tr>
<td>Dopamine</td>
<td>8.13 ± 0.07</td>
<td>7.41</td>
<td>0.62 ± 0.01</td>
</tr>
<tr>
<td>(+)-7-OH-DPAT</td>
<td>8.07 ± 0.27</td>
<td>7.61</td>
<td>0.78 ± 0.05</td>
</tr>
<tr>
<td>PD 128,907</td>
<td>8.13 ± 0.07</td>
<td>7.41</td>
<td>0.62 ± 0.01</td>
</tr>
</tbody>
</table>

The obtained K$_i$ values were calculated from respective mean pK$_i$ values. hD$_3$/hD$_2$ affinity ratio was obtained by dividing K$_i$ value at hD$_3$ receptors by K$_i$ value at hD$_2$ for agonist ligands ratios show a wide variation depending on whether K$_i$ value at hD$_3$ is compared with K$_i$ or K$_i$ value at hD$_2$.
timal stimulation was observed at NaCl concentrations of 100 and 150 mM for hD2 and hD3 membranes, respectively. 2) GDP concentration dependently reduced basal binding of \[^{35}\text{S}]\text{GTP} \gamma \text{S} to both hD2 and hD3 cell membranes. 3) MgCl\(_2\) increased dopamine-dependent \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding to a maximum at around 3 to 10 mM for both receptor subtypes. 4) Stimulation of \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding was linear with time over the period of the incubations. In view of the lower stimulation of \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding by agonists at hD3 receptors, a higher concentration of \[^{35}\text{S}]\text{GTP} \gamma \text{S} was used (1.0 nM) than with hD2 (0.1 nM) to provide a stronger signal. Typical binding of \[^{35}\text{S}]\text{GTP} \gamma \text{S} (0.1 nM) to CHO-hD2 membranes was 90 to 100 fmol/mg basal and 230 to 250 fmol/mg in the presence of dopamine (10 \(\mu\)M). Typical binding of \[^{35}\text{S}]\text{GTP} \gamma \text{S} (1 nM) to CHO-hD3 membranes was 1000 to 1100 fmol/mg basal and 1500 to 1600 fmol/mg with dopamine (10 \(\mu\)M). In control experiments in which the concentration of \[^{35}\text{S}]\text{GTP} \gamma \text{S} used for hD3 receptors was 0.1 nM (as for hD2 receptors), the pEC\(_{50}\) for dopamine was 8.04 \(\pm\) 0.03 (\(n = 3\)) nM, not significantly different from the pEC\(_{50}\) observed with a \[^{35}\text{S}]\text{GTP} \gamma \text{S} concentration of 1 nM (8.00 \(\pm\) 0.07, Table 4). Dopamine-induced stimulation was 45.8 \(\pm\) 2.8\% (\(n = 3\)).

\[^{35}\text{S}]\text{GTP} \gamma \text{S} Binding at CHO-hD3 and CHO-hD2 Cell Membranes: Agonist Actions. Dopamine, PD 128,907, and (+)-7-OH-DPAT increased \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding to CHO-hD3 and CHO-hD2 membranes in a concentration-dependent manner, with EC\(_{50}\), \(E_{\text{max}}\), and nH values shown in Table 4. S\_14297 exhibited slight agonist actions at hD2 receptors (\(E_{\text{max}} = 20.6\%\)) but no agonist activity was detected at hD3 receptors (Fig. 5). PD 128,907 was almost twice as efficacious at hD2 as at hD3 receptors, whereas (+)-7-OH-DPAT was a partial agonist at both receptor subtypes. The ratios of EC\(_{50}\) values at hD2/hD3 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 hD2 and hD3, we did not observe marked changes in EC\(_{50}\) and \(E_{\text{max}}\) 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}\text{S}]\text{GTP} \gamma \text{S} Binding at CHO-hD3 and CHO-hD2 Cell Membranes: Antagonist Actions. Haloperidol, GR 218,231, and S\_14297 did not alter \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding from basal levels at hD3 receptors or, except S\_14297 (as described above) at hD2 receptors. The inhibition of dopamine-stimulated \[^{35}\text{S}]\text{GTP} \gamma \text{S} 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 \(\pm\) 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 hD3 Receptor Coupling. Membranes were prepared from CHO-hD3 cells treated with PTX (100 ng/ml) or cholera toxin (1 \(\mu\)g/ml) for 6 h. Pretreatment with PTX reduced, but did not totally suppress, dopamine-dependent \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding; it was attenuated by about 80\% (81 \(\pm\) 16 fmol/mg versus 430 \(\pm\) 26 fmol/mg in control), without changes in basal \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding (Fig. 8). The incomplete suppression of dopamine-stimulated \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding was not due to an
insufficiently long incubation of CHO-hD3 cells with PTX. Indeed, when membranes were prepared from CHO-hD3 cells after the 6-h incubation, no subsequent incorporation of $[^35]P$ADP-ribose was observed, indicating that all the $G_{\alpha_{i6}}$ proteins present had already been ADP-ribosylated (Fig. 8).

Dopamine stimulated $[^35]S\text{GTP}\gamma S$ binding to membranes of CHO-hD3 cells treated with cholera toxin with a pEC$_{50}$ of 8.04 ± 0.08 (n = 4), similar to that observed in control membranes (pEC$_{50}$ = 8.00, Table 4), but basal $[^35]S\text{GTP}\gamma S$ 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 $[^35]S\text{GTP}\gamma S$ binding was unchanged (407 ± 61 fmol/mg versus 430 ± 26 fmol/mg in control membranes) (Fig. 8).

**Effect of Antibodies on hD3 and hD2 Receptor Coupling.** The presence of $G_{\alpha_{i6}}$, $G_{\alpha_{i11}}$, and $G_{\alpha_{i1}}$ in both CHO-hD3 and CHO-hD2 cell membranes was demonstrated by immunodetection with specific antibodies (Fig. 9). Preincubation of hD3 and hD2 cell membranes with anti-$G_{\alpha_{i6}}$ subunit antisera significantly (P < .05, Student’s paired t test) attenuated dopamine-dependent $[^35]S\text{GTP}\gamma S$ binding, at both hD3 and hD2 receptors (Fig. 9). Anti-$G_{\alpha_{i11}}$ antisera significantly attenuated dopamine-dependent $[^35]S\text{GTP}\gamma S$ binding to CHO-hD3 but not CHO-hD2 membranes (P < .05, Student’s paired t test). Antisera directed against $G_{\alpha_{i}}$ and an unrelated target, JNK1, did not affect $[^35]S\text{GTP}\gamma S$ binding at either receptor (data not shown).

**Discussion**

The primary purpose of the present study was to investigate the G protein coupling of dopamine hD3 receptors. The results demonstrate that hD3 (and hD2) receptors mediate stimulation of $[^35]S\text{GTP}\gamma S$ 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 hD3 and hD2 sites.

First, despite the 11-fold higher hD3 receptor expression level (15 pmol/mg), the dopamine-elicited increase in $[^35]S\text{GTP}\gamma S$ binding (up to 1.6-fold) was less than that at hD2 receptors. Partial inactivation of hD3 receptors using the alkyllating agent EEDQ showed that high hD3 receptor expression levels are necessary for stimulation of G protein activation, because EEDQ treatment reduced the stimulation of $[^35]S\text{GTP}\gamma S$ 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 $[^35]S\text{GTP}\gamma S$ binding by dopamine and is consistent with the 5-fold $K_{i}$ ratio for activation of hD3 receptors (see Results). Thus the limited stimulation of $[^35]S\text{GTP}\gamma S$ binding to CHO-hD3 membranes appears to be a property of hD3 receptors themselves and not due to insufficient intrinsic efficacy of dopamine. It should be noted that the modest stimulation at hD3 receptors is not a consequence of augmented basal $[^35]S\text{GTP}\gamma S$ binding, because basal $[^35]S\text{GTP}\gamma S$ binding was unaffected by receptor inactivation (not shown). Furthermore, the low-fold stimulation in CHO-hD3 membranes is unlikely to be due to a global lack of activatable G proteins: the amount ($B_{\text{max}}$) of dopamine-activated G proteins in CHO-hD3 cell membranes is about 3-fold higher than that in CHO-hD2 cell membranes (Table 1 and Fig. 1). Taken together, the present data suggest that stimulation of hD3 receptors less effectively induces the conformational changes necessary for G protein activation than at hD2 receptors (Chio et al., 1994), perhaps due to a slower rate of G protein coupling/uncoupling at hD3 receptors or, alternatively, to interaction with different G protein subtypes at hD3 versus hD2 receptors, a possibility discussed below.

Second, agonist efficacy varied between hD2 and hD3 receptors. S 14297, previously characterized as a D3 receptor antagonist in vivo (Millan et al., 1995a,b) exhibited residual intrinsic activity at hD2 receptors (Table 4) but no detectable agonist activity at hD3 receptors. In the present high-expressing CHO-hD3 cell membranes, it might have been expected that partial agonist actions at hD3 receptors would be “amplified” to yield maximal activation of $[^35]S\text{GTP}\gamma S$ binding, like dopamine. Nevertheless, both (+)-7-OH-DPAT and PD 128,907 behaved as partial agonists at hD3 receptors (Table 4 and Fig. 5). It therefore appears that despite the high levels of hD3 receptors, the high R/G ratio in CHO-hD3 membranes, and, as discussed above, receptor reserve for activation by dopamine, the present $[^35]S\text{GTP}\gamma S$ binding methodology more readily differentiates partial agonist efficacies at hD3 receptors than certain downstream models of hD3 (or hD2) 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 hD3 versus hD2 receptors was greater than previously reported (Levant, 1997). (+)-7-OH-DPAT displayed an hD3/hD2 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

<table>
<thead>
<tr>
<th>Agonist</th>
<th>hD3</th>
<th>hD2</th>
<th>EC$_{50}$</th>
<th>EC$_{50}$</th>
<th>E$_{MAX}$</th>
<th>nH</th>
<th>EC$_{50}$</th>
<th>EC$_{50}$</th>
<th>E$_{MAX}$</th>
<th>nH</th>
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<tr>
<td>Dopamine</td>
<td>6.45 ± 0.03</td>
<td>350</td>
<td>103.5 ± 4.9</td>
<td>1.10 ± 0.10</td>
<td>8.00 ± 0.07</td>
<td>10</td>
<td>100.1 ± 3.2</td>
<td>0.82 ± 0.07</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>(+)-7-OH-DPAT</td>
<td>7.35 ± 0.11</td>
<td>44.6</td>
<td>64.5 ± 2.8</td>
<td>0.86 ± 0.10</td>
<td>9.24 ± 0.26</td>
<td>0.58</td>
<td>55.4 ± 4.2</td>
<td>1.27 ± 0.24</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>PD 128,907</td>
<td>6.33 ± 0.10</td>
<td>487</td>
<td>116 ± 3.8</td>
<td>0.73 ± 0.18</td>
<td>8.91 ± 0.12</td>
<td>1.22</td>
<td>63.6 ± 2.8</td>
<td>1.21 ± 0.31</td>
<td>382</td>
<td></td>
</tr>
<tr>
<td>S 14297</td>
<td>7.31 ± 0.08</td>
<td>48.6</td>
<td>20.6 ± 1.9</td>
<td>1.53 ± 1.11</td>
<td>n.c.</td>
<td>n.c.</td>
<td>63.6 ± 2.8</td>
<td>1.21 ± 0.31</td>
<td>n.c.</td>
<td></td>
</tr>
</tbody>
</table>

n.c., not computable.
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, $[^{35}S]GTP_\gamma S$ 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 hD3 and hD2 receptors corresponded to the order of affinity determined in competition binding experiments, although a reduced preference for hD3 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 hD3 or hD2 receptors at concentrations up to $10^{-5}$ M. At higher concentrations, $[^{35}S]GTP_\gamma S$ 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 hD3 receptors expressed in CHO cells (Malmberg et al., 1998) reported that basal $[^{35}S]GTP_\gamma S$ 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 ($>10^{-6}$ 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 $[^{35}S]GTP_\gamma S$ binding both in the present study and that of Malmberg et al. (1998) (high concentrations of GDP and NaCl) favor suppression of constitutive hD3 and hD2 receptor activation (Gardner et al., 1996). Indeed, some studies reported that haloperidol shows negative efficacy in models of D3 and D2 receptor activation (mitogenesis, Griffon et al., 1996; prolactin secretion, Nilsson et al., 1996).

Fourth, G protein activation by dopamine at hD3 receptors is PTX sensitive (Fig. 8), implicating G i/o G proteins. This is analogous to the known PTX sensitivity of D2 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 hD3 and hD2 receptors in antibody tests. In the present study, dopamine-

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**Fig. 5.** Agonist stimulation of hD3 and hD2 receptor-mediated G protein activation. $[^{35}S]GTP_\gamma S$ binding is expressed as a percentage of maximal stimulation given by dopamine. A, fold stimulation of $[^{35}S]GTP_\gamma S$ binding by dopamine at hD2 and hD3 receptors. B, agonist concentration-response curves at hD2 receptors. C, agonist concentration-response curves at hD3 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. $E_{\text{max}}$, $pEC_{\text{50}}$, and nH data from these experiments are shown in Table 4.

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**Table 5**

Antagonism of dopamine-stimulated $[^{35}S]GTP_\gamma S$ binding to CHO-hD2 and -hD3 membranes

Antagonist potencies ($pK_B$ values) were calculated from IC$_{50}$ values for inhibition of dopamine (3 μM for hD2, 1 μM for hD3)-stimulated $[^{35}S]GTP_\gamma S$ 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.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>hD2</th>
<th>hD3</th>
<th>$K_B^{hD2/hD3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pK_B$</td>
<td>$K_B$</td>
<td>$pK_B$</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>9.30 ± 0.13</td>
<td>0.50</td>
<td>7.67 ± 0.27</td>
</tr>
<tr>
<td>S 14297</td>
<td>6.56 ± 0.09</td>
<td>275</td>
<td>7.53 ± 0.08</td>
</tr>
<tr>
<td>GR 218231</td>
<td>7.17 ± 0.16</td>
<td>67.6</td>
<td>8.65 ± 0.03</td>
</tr>
</tbody>
</table>
stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding at hD$_3$ and hD$_2$ receptors was inhibited by an antiserum that recognizes the three $\alpha_i$ subunits ($\alpha_i1$/$\alpha_i2$/$\alpha_i3$) and, more weakly, $\alpha_O$ subunits (Cussac et al., 1996). This antiserum inhibited dopamine-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ 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$_{\alpha_{i1}}$ antibodies (Izenwasser and Côte, 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 G$_\alpha$ 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 $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ 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$_{\alpha_O}$ proteins in CHO-hD$_3$ cells, in agreement with previous studies in pituitary cells (Cussac et al., 1996). Thus, a component of dopamine-dependent $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ 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 $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ 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 $\alpha_S$) subunits are expressed in both cell lines (Fig. 9). Furthermore, the inhibition by anti-G$_{\alpha_{q/11}}$ antibodies is not simply due to non-specific Ig interactions, because the same concentration of antisera against an unrelated target (JNK1) did not affect dopamine-dependent $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ 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 $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ 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 $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ 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-
involved in hD3 receptor coupling and their relevance to physiological actions require further investigation.

Acknowledgment

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References


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Hall DA and Strange PG (1997) Evidence that antipsychotic drugs are inverse agonists at the dopamine D3 receptor. J Neurosci 17:1163–1175.


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Fig. 8. Partial attenuation of dopamine-stimulated [35S]GTPyS binding by PTX, but not cholerina toxin. A, [32P]ADP-ribose incorporation catalyzed by PTX. ADP-ribosylation of CHO-hD3 cell membranes preincubated with or without PTX or cholerina toxin (CT) for 6 h was carried out as described in Materials and Methods. [32P]ADP-ribosylated G0i proteins were revealed by a 6-h exposure to Hyperfilm. The data shows that PTX pretreatment abolished subsequent [32P]ADP-ribose incorporation. The same result was obtained in a second, independent experiment. B, Effect of PTX and CT on dopamine-stimulated [35S]GTPyS binding. CHO-hD3 cell were incubated for 6 h with PTX or CT and stimulation of [35S]GTPyS 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 femtomoles per milligram of dopamine-induced [35S]GTPyS binding.

Fig. 9. Inhibition by anti-G protein antibodies of dopamine-stimulated [35S]GTPyS binding to CHO-hD2 and CHO-hD3 cell membranes. A, immunodetection of G0i, G0s and G0q subunits in both CHO-hD2 and CHO-hD3 cell membranes was performed as described in Materials and Methods. B, CHO-hD2 and CHO-hD3 cell membranes were incubated for 5 h at 4°C in the presence of antibodies used in A. Stimulation of [33S]GTPyS 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 [33S]GTPyS binding observed in control (untreated) samples. *P < .05, **P < .01 versus control (2-tailed, paired t test).


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