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# G-Protein Sensitivity of Ligand Binding to Human Dopamine $D_2$ and $D_3$ Receptors Expressed in *Escherichia coli*: Clues for a Constrained $D_3$ Receptor Structure[1]

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## ABSTRACT

Human dopamine $D_2$ and $D_3$ receptors were expressed in Chinese hamster ovary (CHO) and *Escherichia coli* cells to compare their ligand binding properties in the presence or absence of G-proteins and to analyze their ability to interact with $G_{i/o}$-proteins. Binding affinities of agonists (dopamine, 7-OH-DPAT, PD128907, lisuride) and antagonists/inverse agonists (haloperidol, risperidone, domperidone, spiperone, raclopride, nemonapride), measured using [$^{125}$I]iodosulpride and [$^3$H]7-OH-DPAT, were similar for $hD_3$ receptors in *E. coli* and CHO cell membranes. Both agonists and antagonists showed 2- to 25-fold lower binding affinities at $hD_2$ receptors in *E. coli* versus CHO cell membranes (measured with [$^3$H]spiperone), but the rank order of potencies remained similar. Purported inverse agonists did not display higher affinities for G-protein-free receptors. In CHO membranes, GppNHp decreased high affinity agonist ([$^3$H]7-OH-DPAT) binding at $hD_2$ receptors but not at $hD_3$ receptors. Also, [$^3$H]7-OH-DPAT (nanomolar concentration range) binding was undetectable at $hD_2$ but clearly measurable at $hD_3$ receptors in *E. coli* membranes. Addition of a $G_{i/o}$-protein mix to *E. coli* membranes increased high affinity [$^3$H]7-OH-DPAT binding in a concentration-dependent manner at $hD_2$ and $hD_3$ receptors; this effect was reversed by addition of GppNHp. The potency of the $G_{i/o}$-protein mix to reconstitute high affinity binding was similar for $hD_2$ and $hD_3$ receptors. Thus, agonist binding to $D_3$ receptors is only slightly affected by G-protein uncoupling, pointing to a rigid receptor structure. Furthermore, we propose that the generally reported lower signaling capacity of $D_3$ receptors (versus $D_2$ receptors) is not due to its lower affinity for G-proteins but attributed to its lower capacity to activate these G-proteins.

Dopamine receptors belong to the superfamily of G-protein-coupled receptors. Five dopamine receptors have been found and classified into the D1-like family ($D_1$ and $D_5$ receptors) and the D2-like family ($D_2$, $D_3$, and $D_4$ receptors) (Missale et al., 1998). Receptor genes of the D1-like family do not contain introns, whereas members of the D2-like family possess multiple introns. For instance, two splice variants of the $D_2$ receptor have been found; the $D_{2short}$ ($D_{2S}$) receptor, lacking a 29-amino acid insert in the third cytoplasmic loop, and the $D_{2long}$ ($D_{2L}$) receptor (Monsma et al., 1989). Differences in G-protein coupling of these splice variants have been reported (Picetti et al., 1997), but the physiological relevance of these splice variants remains unclear.

Human dopamine $D_2$ and $D_3$ receptors have an overall amino acid sequence similarity of 52%, which increases to 78% if only transmembrane regions are considered (Giros et al., 1990). Most ligands that bind to $hD_2$ receptors also bind to $hD_3$ receptors and their rank order of potency is quite similar. In general, dopamine agonists have a higher affinity for $hD_3$ receptors, whereas antagonists have a slightly higher affinity for $hD_2$ receptors. The pharmacological properties of the $hD_2$ receptor splice variants are almost identical (Liu et al., 1992; Leysen et al., 1993; Schotte et al., 1996). Although both $hD_2$ and $hD_3$ receptors are thought to couple to $G_{i/o}$ proteins, this is much less clear for the $hD_3$ than for the $hD_2$ receptor. Guanine nucleotide modulation of agonist binding

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**ABBREVIATIONS:** CHO, Chinese hamster ovary; 7-OH-DPAT, (+)-7-(dipropylamino)-5,6,7,8-tetrahydro-2-naphthalenol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; G-protein, heterotrimeric guanine nucleotide binding protein; GppNHp, guanylyl imidodiphosphate; $hD_2$, human dopamine $D_2$; $hD_3$, human dopamine $D_3$; $pIC_{50}$, $-\log IC_{50}$ (concentration of the compound producing 50% inhibition of the specific binding of the radioactive ligand); $pEC_{50}$, $-\log EC_{50}$ (concentration of the compound producing 50% effect).

at $hD_2$ receptors has been clearly demonstrated, whereas conflicting results have been obtained for $hD_3$ receptors (Sokoloff et al., 1992; Freedman et al., 1994; MacKenzie et al., 1994; Tang et al., 1994; Akunne et al., 1995; McAllister et al., 1995). In addition, coupling of $hD_2$ receptors to various pertussis-toxin-sensitive signaling pathways has been reported, whereas a total lack or a weak coupling to these pathways has been described for $hD_3$ receptors (MacKenzie et al., 1994; Tang et al., 1994; McAllister et al., 1995). Only recently, we succeeded in demonstrating clear activation of $hD_3$ receptors at different levels of the signal transduction cascade when expressed at high levels in Chinese hamster ovary (CHO) cells (Vanhauwe et al., 1999).

According to the allosteric ternary complex model (Lefkowitz et al., 1993; Samama et al., 1993), receptors, by themselves, can occur in an active and an inactive state. In the absence of agonist, a fraction of the receptor population can exist in an active state, leading to basal or constitutive activity. Agonist binding increases the population of active receptors. In contrast, binding of inverse agonists drives the receptor to the inactive state, diminishing the basal activity. It is not yet clear whether agonist binding recruits G-proteins to the activated receptor or that G-protein coupling promotes subsequent agonist binding. Probably, both processes play in concert (ternary complex formation). However, it is generally found that agonists bind with higher affinity to G-protein-coupled receptors than to uncoupled receptors, whereas neutral antagonists do not distinguish between G-protein-coupled and uncoupled receptors. Furthermore, it was suggested that inverse agonists have a higher affinity for uncoupled than for G-protein-coupled receptors (Costa et al., 1992). Inverse agonists have been identified for $D_2$ (Hall and Strange, 1997; Kozell and Neve, 1997; Vanhauwe et al., 2000) and $D_3$ receptors (Griffon et al., 1996; Malmberg et al., 1998), indicating that both receptors can have basal activity.

To demonstrate clearly the effect of G-protein receptor coupling on ligand binding, one would need the receptor in a G-protein-free environment. *Escherichia coli* cells, which do not contain G-proteins, provide a system for producing uncoupled receptors. Certain G-protein-coupled receptors have been successfully expressed in *E. coli* (Marullo et al., 1990; Freissmuth et al., 1991; Bertin et al., 1992; Grisshammer et al., 1993; Munch et al., 1995).

In this study, we succeeded for the first time to express $hD_{2S}$, $hD_{2L}$, and $hD_3$ receptors in *E. coli*. To scrutinize the difference in G-protein-coupling properties of $hD_{2S}$, $hD_{2L}$, and $hD_3$ receptors, we compared the binding properties of agonists and antagonists for recombinant receptors expressed in *E. coli* versus CHO cells. Measurements on recombinant $hD_2$ and $hD_3$ receptors in *E. coli* membranes were carried out in the absence or presence of added $G_{i/o}$-proteins. Our study illustrates that agonist binding to $hD_3$ receptors is poorly sensitive to G-protein coupling, whereas $hD_2$ receptor high affinity agonist binding is highly dependent on G-proteins. Nevertheless, $hD_3$ and $hD_2$ receptors were found to have equal affinity for the $G_{i/o}$-protein mix. A constrained $hD_3$ receptor structure that retains a conformation of high affinity agonist binding in the uncoupled and G-protein state is hypothesized, along with a conformation that binds well, but poorly activates G-proteins.

## Experimental Procedures

**Bacterial Expression and Membrane Preparation.** The cDNAs of $hD_{2S}$, $hD_{2L}$, and $hD_3$ receptors were cloned, and the sequence was verified by DNA sequencing (Schotte et al., 1996). For each receptor, the cDNA was ligated in the expression vector pMal-*p* so that it was in-frame with the maltose binding protein (MalE). All plasmid constructs were confirmed by DNA sequencing. The resulting plasmids coded for the MalE protein fused via a polylinker, containing a protease factor Xa cleavage site, to the receptor at the C terminus. Fusion and wild-type plasmids were transformed into competent *E. coli* TB1 cells [araΔ (lac proAB) rpsL (Φ80 lacZΔM15) hsdR] by electroporation and plated on 2xYT plates containing 100 μg/ml ampicillin. A single colony was inoculated into 2 ml of 2xYT medium containing 100 μg/ml ampicillin. After 8 h of growth, the 2-ml starter culture was added to 500-ml 2xYT medium containing 100 μg/ml ampicillin and grown overnight in a shaking incubator (New Brunswick Scientific, Edison, NJ) at 300 rpm and 37°C. The optical density was determined, and the cells were harvested by centrifugation (2000*g* for 10 min at 4°C) and resuspended to an optical density of 1.5 in 2xYT, containing 100 μg/ml ampicillin and 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were further incubated for 4 h at 25°C, harvested by centrifugation, and resuspended in ice-cold 50 mM Tris-HCl, pH 7.4, containing 20% sucrose and 1 freshly dissolved tablet of protease inhibitor cocktail (Complete) per 50 ml. In a first series of experiments on the pharmacological characterization of $hD_3$ receptors, *E. coli* cell suspensions were frozen as pellets and thawed before use. In subsequent studies on the characterization of $D_2$ receptors and addition of G-proteins to *E. coli* membranes, frozen droplets were prepared by freezing drops of the cell suspension in liquid nitrogen followed by storage at −70°C until use. After thawing of the droplets, the cell suspension was passed four times at 800 psi through a French press (Spectronic Instruments, Cheshire, England). The suspension was diluted in 50 mM Tris-HCl, pH 7.4, containing 10% glycerol. After a low spin centrifugation (1400*g* for 5 min at 4°C) for removal of intact cells, the supernatant was collected and centrifuged at 90,000*g* for 1 h at 4°C. The membrane pellet was suspended in 50 mM Tris-HCl, pH 7.4, containing 10% glycerol and used in radioligand binding experiments.

**CHO Cell Culture and Membrane Preparation.** CHO cells expressing the $hD_{2S}$, $hD_{2L}$, or $D_3$ receptors (Vanhauwe et al., 1999) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum in a humidified atmosphere of 5% $CO_2$ at 37°C. Cells were subcultured at 80 to 90% confluence.

Cells were subcultured from 175-$cm^2$ tissue culture flasks to 145-$cm^2$ Petri dishes. At 90% confluence, 5 mM sodium butyrate was added to increase the receptor expression level and the cells were further incubated for 24 h (Palermo et al., 1991). The medium was removed, and the Petri dishes were washed once with 5 ml of ice-cold PBS and stored at −70°C. Cells on Petri dishes were thawed, and 5 ml of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride was added per dish. The cells were harvested and homogenized with a dual homogenizer (motor-driven Teflon pestle and conical glass tube). The homogenate was centrifuged (10 min at 1000*g* at 4°C), and the resulting pellet was resuspended and centrifuged again (10 min at 1300*g* at 4°C). Both supernatants were pooled and centrifuged at 50,000*g* for 1 h at 4°C. The resulting pellet was resuspended in 50 mM Tris-HCl (pH 7.4), containing 10% glycerol and stored in aliquots at −70°C. The protein concentration in membrane preparations was measured with the Bradford protein assay using BSA as a calibration standard.

**Radioligand Binding Assays.** For radioligand binding experiments on $hD_3R$-*E. coli* cells, the cell suspension was thawed and washed twice in the incubation buffer for the ligand. For radioligand binding experiments on *E. coli* or CHO cell membranes, the mem-

branes were thawed and resuspended in the incubation buffer for the ligand.

[$^3$H]7-OH-DPAT was used to measure agonist binding to $hD_3$, $hD_{2S}$, and $hD_{2L}$ receptors. The incubation with [$^3$H]7-OH-DPAT was performed for 30 min at 25°C in a total volume of 0.5 ml, containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM EGTA (for $hD_2$R-*E. coli* membranes, the total incubation volume was 1 ml). [$^{125}$I]Iodosulpride was used to measure antagonist binding to $hD_3$ receptors in *E. coli* cells and CHO cell membranes. The incubation with [$^{125}$I]iodosulpride was performed for 30 min at 25°C in a total volume of 0.25 ml, containing 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 0.1% BSA. [$^3$H]Spiperone was used to measure antagonist binding to $hD_{2S}$ and $hD_{2L}$ receptors in *E. coli* membranes and CHO cell membranes. The incubation with [$^3$H]spiperone was performed for 30 min at 37°C in a total volume of 0.5 ml, containing 50 mM Tris-HCl, pH 7.4, and 120 mM NaCl (for $hD_2$R-*E. coli* membranes the total incubation volume was 1 ml). The amount of protein per incubation was 10 to 20 $\mu$g for *E. coli* membranes and cells and for $hD_{2S}$R-CHO cell membranes. For $hD_3$R-CHO and $hD_{2L}$R-CHO cell membranes 5 to 10 $\mu$g of protein was used. Nonspecific binding was estimated in the presence of 10 $\mu$M haloperidol. The reaction was terminated by filtration through Whatman GF/B filters, presoaked in 0.1% polyethyleneimine. Filters were rinsed twice with 5 ml of ice-cold incubation buffer. The filter-bound radioactivity was measured in a liquid scintillation spectrometer (Tricarb, Packard, Meriden, CT), using 3 ml of scintillation fluid. Specific binding was calculated as the difference between total binding and nonspecific binding. For ligand concentration binding isotherms, [$^3$H]7-OH-DPAT was used at 10 to 12 concentrations in the range of 0.1 to 20 nM for CHO cell membranes and 0.1 to 10 nM for *E. coli* cell membranes (high concentrations of [$^3$H]7-OH-DPAT resulted in high nonspecific binding at *E. coli* membranes), and [$^{125}$I]iodosulpride was used at 10 concentrations in the range of 0.1 to 3 nM and [$^3$H]spiperone was used at 10 to 12 concentrations in the range of 0.01 to 1 nM.

Ligand concentration binding isotherms were fitted to a rectangular hyperbola by nonlinear regression analysis in which the apparent equilibrium dissociation constant ($K_d$) and the maximum number of binding sites ($B_{max}$) were free parameters. Computerized curve fitting was performed using the GraphPad Prism software.

In competition binding experiments at $hD_3$ receptors, serial dilutions of unlabeled compounds were incubated with [$^3$H]7-OH-DPAT (2 nM) or [$^{125}$I]iodosulpride (0.4 nM) and CHO or *E. coli* membranes. In the case of $hD_{2S}$ and $hD_{2L}$ receptors, serial dilutions of unlabeled compounds (10–12 concentrations, range 0.1 mM to 0.1 nM) were incubated with [$^3$H]spiperone (0.5 nM) and CHO or *E. coli* membranes. Competition curves were fit to a sigmoid by nonlinear regression analysis according to algorithms described by Oestreicher and Pinto (1987), in which the $pIC_{50}$ and the Hill coefficient were free parameters. Inhibition constants ($K_i$) were calculated according to Cheng and Prusoff (1973). Assays were run in duplicate in ligand concentration binding isotherms and in singlets in competition binding experiments and repeated in independent experiments ($n$ numbers in tables and figures). Curves were calculated for individual experiments, and the mean of the derived parameters was calculated.

**Reconstitution of High Affinity [$^3$H]7-OH-DPAT Binding with G-Proteins.** A commercially available bovine $G_{i/o}$-protein mix ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\beta\gamma$ purified from bovine brain) was diluted in 50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$, 1 mM EGTA, and 0.15% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) and then 5-fold diluted and mixed with *E. coli* membrane suspension by vortexing. At this concentration of CHAPS, 90% of the binding is retained (results not shown). *E. coli* membranes (30–40 $\mu$g of protein/300 $\mu$l) were incubated in a total volume of 0.5 ml with serial dilutions of the G-protein mix and 0.75 nM or 2 nM [$^3$H]7-OH-DPAT for $hD_3$ and $hD_{2S}$ receptors, respectively. The assay buffer contained 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, and 0.03% CHAPS. After a 30-min incubation period at 25°C, the reaction was terminated by filtration and the amount of bound radioactivity was determined as described above. The concentration of the $G_{i/o}$-protein mix was calculated assuming an average molecular mass of 87,000 Da.

**Materials.** [$^3$H]Spiperone (3.5 TBq/mmol), [$^{125}$I]iodosulpride (74.1 TBq/mmol), and [$^3$H]7-OH-DPAT (5.5 TBq/mmol) were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). The *E. coli* strain (TB1) was from Stratagene (La Jolla, CA). The expression vector pMal-p was purchased from New England BioLabs (Leusden, Netherlands). The $G_{i/o}$-protein mix purified from bovine brain was obtained from Calbiochem (La Jolla, CA).

The 2xYT, Dulbecco's modified Eagle's medium, and fetal bovine serum were from Life Technologies (Gaithersburg, MD). The Bradford protein assay kit was from Bio-Rad (Richmond, CA). PD128907 and dopamine were purchased from Research Biochemicals International (Natick, MA). Raclopride and nemonapride were from Astra Arcus (Stockholm, Sweden) and Yamanouchi (Tokyo, Japan), respectively. TL99 was from ICN Pharmaceuticals (Costa Mesa, CA). Haloperidol, domperidone, risperidone, and spiperone are original products of Janssen Pharmaceutica (Beerse, Belgium). 7-OH-DPAT (racemic mixture) was synthesized in-house. Ampicillin, Complete protease inhibitor cocktail, CHAPS, isopropyl-$\beta$-D-thiogalactopyranoside, and 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride were purchased from Boehringer Mannheim (Mannheim, Germany). All other reagents were analytical grade from Merck (Haasrode, Belgium) or Sigma (St. Louis, MO). GF/B glass-fiber filters were from Whatman (Kent, UK). The scintillation fluid (Ultima Gold MV) was from Packard (Meriden, CT). The GraphPad Prism program was from GraphPad Software, Inc. (San Diego, CA). Dopamine, 7-OH-DPAT, and PD128907 were dissolved and diluted in assay buffer. Lisuride and TL99 were dissolved and diluted in ethanol. Haloperidol, spiperone, domperidone, risperidone, raclopride, and nemonapride were dissolved and diluted in dimethyl sulfoxide (DMSO). For compounds that were dissolved and diluted in DMSO or ethanol, a last dilution step of 20-fold in assay buffer was performed just before addition to the assay mixture in which the dilution was 10-fold. In control assays, ethanol or DMSO was added to a final concentration of 0.5%.

## Results

**Ligand Concentration Binding Isotherms at $hD_3$ Receptors.** Ligand concentration binding isotherms on $hD_3$ receptors were measured with the partial agonist [$^3$H]7-OH-DPAT and the antagonist [$^{125}$I]iodosulpride on *E. coli* cells and CHO cell membranes expressing the recombinant receptor (Vanhauwe et al., 1999). The ligand concentration binding curve of [$^{125}$I]iodosulpride on *E. coli* cells is shown in Fig. 1A. $B_{max}$ and apparent $K_d$ values obtained with $hD_3$R-*E. coli* cells and $hD_3$R-CHO cell membranes are shown in Table 1A. The affinity of [$^{125}$I]iodosulpride for $hD_3$ receptors expressed in *E. coli* or CHO cells was similar ($K_d$ = 1.2–1.4 nM). Also, the agonist [$^3$H]7-OH-DPAT bound with comparable affinity to $hD_3$R-*E. coli* cells and $hD_3$R-CHO cell membranes ($K_d$ = 1.5 nM). The affinity of [$^3$H]7-OH-DPAT for $hD_3$R-CHO cell membranes was not affected by addition of GppNHp. No binding could be detected in vector-transformed *E. coli* cells and CHO cell membranes with either radioligand.

**Ligand Concentration Binding Isotherms for $hD_{2S}$ and $hD_{2L}$ Receptors.** Ligand concentration binding isotherms on $hD_2$ receptors were measured with the partial agonist [$^3$H]7-OH-DPAT and the antagonist [$^3$H]spiperone. To improve the signal-to-noise ratio, membranes were prepared from *E. coli* cells expressing $hD_{2S}$ or $hD_{2L}$ receptors.

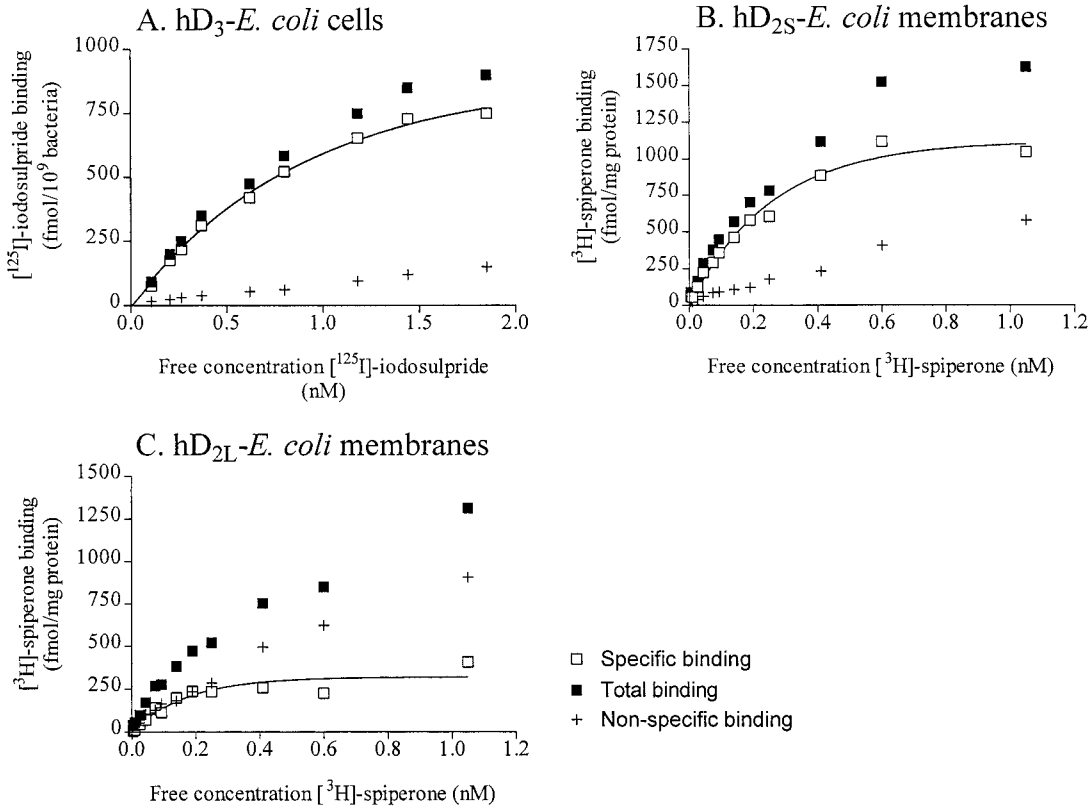


**Fig. 1.** Antagonist radioligand binding isotherms using [$^{125}$I]iodosulpride at $hD_3$-*E. coli* cells (A) and [$^3$H]spiperone at $hD_{2S}$-*E. coli* (B) or $hD_{2L}$-*E. coli* (C) cell membranes. Experiments are performed as described under *Experimental Procedures*. Representative curves are shown in which each point was determined in duplicate. Mean $K_d$ and $B_{max}$ values of three to six experiments are shown in Table 1.

The ligand concentration binding curves of [$^3$H]spiperone on $hD_{2S}$R- and $hD_{2L}$R-*E. coli* membranes are shown in Fig. 1, B and C, respectively. $B_{max}$ and apparent $K_d$ values are listed in Table 1B. The antagonist [$^3$H]spiperone bound with a similar affinity to $hD_{2S}$ and $hD_{2L}$ receptors in *E. coli* than in CHO cell membranes (Student's $t$ test, $P > .05$; $K_d$ = 0.1–0.2 nM)). [$^3$H]7-OH-DPAT binding was undetectable in $hD_{2S}$R-*E. coli* or $hD_{2L}$R-*E. coli* cell membranes within the tested concentration range (0.1–10 nM). In contrast, [$^3$H]7-OH-DPAT bound to $hD_{2S}$R- and $hD_{2L}$R-CHO cell membranes; its affinity was similar for either splice variant ($K_d$ = 2.3–2.9 nM). Addition of GppNHp significantly decreased by nearly 4-fold the affinity of [$^3$H]7-OH-DPAT for $hD_{2S}$ (Student's $t$ test, $P < .005$) and $hD_{2L}$ (Student's $t$ test, $P < .05$) receptors in CHO cell membranes.

**Inhibition Binding Experiments at $hD_3$ Receptors.** The agonists dopamine, 7-OH-DPAT, PD128907, TL99, and lisuride and the presumed antagonists haloperidol, spiperone, domperidone, risperidone, raclopride, and nemonapride were tested for their potency to inhibit [$^{125}$I]iodosulpride and [$^3$H]7-OH-DPAT binding to $hD_3$ receptors. The $pIC_{50}$ and $K_i$ values are listed in Table 2. In general, the apparent binding affinities of the antagonists for $hD_3$ receptors expressed in *E. coli* versus CHO cells were similar when using [$^{125}$I]iodosulpride or [$^3$H]7-OH-DPAT. The apparent affinities of agonists were comparable for $hD_3$ receptors expressed in *E. coli* or CHO cells, when tested with [$^{125}$I]iodosulpride. When [$^3$H]7-OH-DPAT was used, dopamine and 7-OH-DPAT were significantly (5–7-fold) less potent at *E. coli* than at CHO cell membranes (paired Student's $t$ test, $P < .05$); no significant decrease in potency could be found for PD128907, TL99, and lisuride in *E. coli* cells (paired Student's $t$ test, $P > .05$). The rank order of potencies of agonists and antagonists was quite similar in $hD_3$R-*E. coli* and $hD_3$R-CHO cell membranes. In $hD_3$R-*E. coli* cells and CHO membranes, raclopride and nemonapride showed lower apparent binding affinities with [$^3$H]7-OH-DPAT than with [$^{125}$I]iodosulpride. It should be noted that both compounds are benzamides like [$^{125}$I]iodosulpride; close structural similarities between nonlabeled and labeled ligands often leads to more effective inhibition.

**Inhibition Binding Experiments at $hD_{2S}$ and $hD_{2L}$ Receptors.** The same series of agonists and antagonists were tested for their potency to inhibit [$^3$H]spiperone binding; $pIC_{50}$ and $K_i$ values are listed in Table 3. The rank order of antagonist potencies was similar for $hD_{2S}$ and $hD_{2L}$ receptors when expressed in *E. coli* or CHO cell membranes. The apparent binding affinities of antagonists for $hD_{2L}$ receptors in *E. coli* membranes were two to five times lower than those in CHO cell membranes. Some compounds showed higher differences with the $hD_{2S}$ receptor. Dopamine and 7-OH-DPAT were about 10 to 20 times less potent in *E. coli* membranes than in CHO cell membranes; PD128907 and lisuride were only three times less potent at $hD_2$-*E. coli* than at $hD_2$-CHO cell membranes. In CHO cell membranes, the potencies of compounds at $hD_{2S}$ and $hD_{2L}$ receptors were virtually similar. This was also the case for these splice variants when expressed in *E. coli* (except for raclopride).

**Reconstitution of G-Protein Interaction with $hD_{2S}$ and $hD_3$ Receptors in *E. coli*.** In a first series of experiments (see Fig. 2), radioligand binding isotherms were deter-

TABLE 1

Binding affinities ($K_d$ values in nM) derived from radioligand concentration binding isotherms with [$^{125}$I]iodosulpride (25°C), [$^3$H]7-OH-DPAT (25°C), or [$^3$H]spiperone (37°C) on $hD_3$ (A) or $hD_{2S/L}$ (B) receptors expressed in *E. coli* and CHO cell membranes

The assay conditions for each radioligand are described under *Experimental Procedures*. Each value represents the mean of $n$ experiments ± S.D. ($n$ numbers between brackets), in which each point was determined in duplicate. Nonspecific binding was determined in the presence of 10 $\mu$M haloperidol. Significant differences are indicated in superscript and explained below the table.

| Compound | Radioligand | | | | | |
|---|---|---|---|---|---|---|
| | $hD_3$-*E. coli* | | | $hD_3$-CHO | | |
| | $K_d$ | $B_{max}$ | $n$ | $K_d$ | $B_{max}$ | $n$ |
| | *nM* | *fmol/10$^9$ bacteria* | | *nM* | *fmol/mg protein* | |
| A) | | | | | | |
| [$^{125}$I]Iodosulpride | 1.2 ± 0.1 | 580 ± 80 | 3 | 1.4 ± 0.1 | 5,100 ± 1,500 | 6 |
| [$^3$H]7-OH-DPAT | 1.5 ± 0.3 | 620 ± 160 | 7 | 1.5 ± 0.4 | 4,800 ± 1,800 | 6 |
| [$^3$H]7-OH-DPAT + GppNHp | N.D. | N.D. | | 1.5 ± 0.6 | 5,600 ± 1,700 | 6 |

| Compound | Radioligand | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $hD_{2S}$-*E. coli* | | | $hD_{2S}$-CHO | | | $hD_{2L}$-*E. coli* | | | $hD_{2L}$-CHO | | |
| | $K_d$ | $B_{max}$ | $n$ | $K_d$ | $B_{max}$ | $n$ | $K_d$ | $B_{max}$ | $n$ | $K_d$ | $B_{max}$ | $n$ |
| | *nM* | *fmol/mg protein* | | *nM* | *fmol/mg protein* | | *nM* | *fmol/mg protein* | | *nM* | *fmol/mg protein* | |
| B) | | | | | | | | | | | | |
| [$^3$H]Spiperone | 0.21 ± 0.02 | 1,000 ± 200 | 5 | 0.14 ± 0.03 | 13,800 ± 2,500 | 3 | 0.19 ± 0.05 | 250 ± 40 | 4 | 0.10 ± 0.03 | 14,200 ± 1,600 | 3 |
| [$^3$H]7-OH-DPAT | N.B.D. | N.B.D. | | 2.3 ± 0.4 | 5,000 ± 1,200 | 3 | N.B.D. | N.B.D. | | 2.9 ± 1.0 | 4,200 ± 1,700 | 4 |
| [$^3$H]7-OH-DPAT + GppNHp | N.D. | N.D. | | 8.2 ± 1.9[a] | 1,200 ± 100 | 3 | N.D. | N.D. | | 11.2 ± 3.3[a] | 1,700 ± 500 | 3 |

N.D., not determined; N.B.D., no binding detectable.

[a] The affinity of [$^3$H]7-OH-DPAT was significantly different from measurements without 100 $\mu$M GppNHp (paired Student's $t$ test, $P < .05$).

TABLE 2

Binding affinities ($pIC_{50}$ mean ± S.D. and apparent $K_i$ value, $n$) of agonists and antagonists at $hD_3$ receptors in *E. coli* cells and CHO cell membranes, derived from competition binding experiments using 0.4 nM [$^{125}$I]iodosulpride or 2 nM [$^3$H]7-OH-DPAT at 25°C

Significant differences are indicated in superscript and explained below the table.

| | [$^{125}$I]Iodosulpride | | | | | | [$^3$H]7-OH-DPAT | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CHO | | | *E. coli* | | | CHO | | | *E. coli* | | |
| | $pIC_{50}$ | $K_i$ | $n$ | $pIC_{50}$ | $K_i$ | $n$ | $pIC_{50}$ | $K_i$ | $n$ | $pIC_{50}$ | $K_i$ | $n$ |
| | *−logM* | *nM* | | *−logM* | *nM* | | *−logM* | *nM* | | *−logM* | *nM* | |
| Agonists | | | | | | | | | | | | |
| Dopamine | 6.65 ± 0.20 | 170 | 6 | 6.53 ± 0.25 | 220 | 3 | 7.54 ± 0.06 | 10.7 | 3 | 6.80 ± 0.23 | 70.7[a] | 6 |
| TL99 | 7.90 ± 0.02 | 9.8 | 3 | 7.98 ± 0.22 | 7.9 | 6 | 8.78 ± 0.09 | 0.63 | 3 | 8.67 ± 0.22 | 1.1 | 4 |
| PD128907 | 8.04 ± 0.02 | 7.1 | 4 | 7.83 ± 0.11 | 11.1 | 3 | 7.94 ± 0.09 | 4.3 | 4 | 7.48 ± 0.35 | 14.8 | 3 |
| 7OH-DPAT | 8.28 ± 0.19 | 4.1 | 4 | 8.15 ± 0.24 | 5.3 | 5 | 8.38 ± 0.10 | 1.6 | 3 | 7.79 ± 0.26 | 7.2[a] | 6 |
| Lisuride | 8.98 ± 0.27 | 0.81 | 4 | 9.45 ± 0.26 | 0.27 | 5 | 9.45 ± 0.11 | 0.13 | 5 | 9.67 ± 0.24 | 0.1 | 3 |
| Antagonists | | | | | | | | | | | | |
| Haloperidol | 7.60 ± 0.19 | 19.4 | 4 | 7.30 ± 0.30 | 37.2 | 5 | 8.02 ± 0.20 | 3.6 | 4 | 7.21 ± 0.32 | 27.7 | 6 |
| Domperidone | 7.60 ± 0.20 | 19.4 | 3 | 7.51 ± 0.20 | 23.3 | 4 | 7.49 ± 0.18 | 12.1 | 4 | 7.18 ± 0.28 | 29.1 | 5 |
| Risperidone | 7.86 ± 0.29 | 10.9 | 6 | 8.01 ± 0.22 | 7.4 | 5 | 7.70 ± 0.16 | 7.4 | 3 | 7.68 ± 0.35 | 9.3 | 4 |
| Raclopride | 8.45 ± 0.09 | 2.8[b] | 5 | 8.51 ± 0.18 | 2.3[b] | 4 | 6.84 ± 0.25 | 53 | 5 | 6.49 ± 0.26 | 150 | 6 |
| Spiperone | 8.90 ± 0.07 | 0.98 | 5 | 8.73 ± 0.32 | 1.4 | 6 | 9.07 ± 0.18 | 0.32 | 5 | 8.61 ± 0.23 | 1.1 | 6 |
| Nemonapride | 9.31 ± 0.18 | 0.38[b] | 5 | 9.57 ± 0.29 | 0.2[b] | 4 | 8.50 ± 0.29 | 1.2 | 4 | 8.56 ± 0.15 | 1.2 | 5 |

[a] The affinity of the compound for $hD_3$ receptors was significantly lower in *E. coli* than in CHO membranes ($P < .05$).
[b] The affinity of the compound was significantly lower when tested with [$^3$H]7-OH-DPAT (versus [$^{125}$I]iodosulpride) ($P < .05$).

TABLE 3

Binding affinities ($pIC_{50}$ mean ± S.D. and apparent $K_i$ value, $n$) of agonists and antagonists at $hD_{2S}$ and $hD_{2L}$ receptors in *E. coli* membranes and CHO cell membranes, derived from competition binding experiments using 0.5 nM [$^3$H]spiperone

| | $hD_{2S}$ Receptor | | | | | | $hD_{2L}$ Receptor | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CHO | | | *E. coli* | | | CHO | | | *E. coli* | | |
| | $pIC_{50}$ | $K_i$ | $n$ | $pIC_{50}$ | $K_i$ | $n$ | $pIC_{50}$ | $K_i$ | $n$ | $pIC_{50}$ | $K_i$ | $n$ |
| | *−logM* | *nM* | | *−logM* | *nM* | | *−logM* | *nM* | | *−logM* | *nM* | |
| Agonists | | | | | | | | | | | | |
| PD128907 | 4.59 ± 0.13 | 3900 | 3 | 4.39 ± 0.05 | 11600 | 3 | 4.46 ± 0.24 | 6000 | 3 | 4.30 ± 0.13 | 7600 | 3 |
| 7OH-DPAT | 5.19 ± 0.22 | 1000 | 3 | 4.50 ± 0.15 | 9100[a] | 3 | 5.14 ± 0.03 | 900 | 3 | 4.20 ± 0.25 | 18000[a] | 3 |
| Dopamine | 5.60 ± 0.19 | 400 | 5 | 4.46 ± 0.25 | 9800[a] | 6 | 5.58 ± 0.18 | 400 | 5 | 4.60 ± 0.18 | 7200[a] | 5 |
| Lisuride | 7.96 ± 0.26 | 1.7 | 3 | 7.69 ± 0.24 | 5.8 | 3 | 8.11 ± 0.20 | 1.4 | 3 | 7.81 ± 0.23 | 4.4 | 3 |
| Antagonists | | | | | | | | | | | | |
| Raclopride | 7.55 ± 0.28 | 4.4 | 4 | 6.41 ± 0.08 | 110[a] | 3 | 7.49 ± 0.12 | 4.9 | 4 | 6.22 ± 0.18 | 170[a] | 3 |
| Risperidone | 7.87 ± 0.16 | 2.3 | 3 | 7.53 ± 0.16 | 8.4 | 4 | 7.76 ± 0.16 | 2.7 | 4 | 7.47 ± 0.23 | 9.8 | 4 |
| Nemonapride | 8.36 ± 0.23 | 0.67 | 3 | 8.09 ± 0.29 | 1.3 | 4 | 8.44 ± 0.23 | 0.55 | 4 | 8.28 ± 0.29 | 0.8 | 4 |
| Domperidone | 8.57 ± 0.20 | 0.41 | 3 | 7.74 ± 0.19 | 5.2[a] | 4 | 8.23 ± 0.30 | 1 | 4 | 7.94 ± 0.13 | 3.3 | 4 |
| Haloperidol | 8.58 ± 0.20 | 0.41 | 4 | 7.60 ± 0.14 | 7.2[a] | 4 | 8.42 ± 0.11 | 0.58 | 4 | 7.96 ± 0.22 | 3.2[a] | 4 |
| Spiperone | 9.07 ± 0.05 | 0.13 | 3 | 9.01 ± 0.10 | 0.28 | 4 | 9.13 ± 0.14 | 0.1 | 4 | 9.05 ± 0.26 | 0.26 | 3 |

[a] The affinity of the compound for $hD_{2S/L}$ receptors was significantly lower in *E. coli* than in CHO membranes ($P < .05$).

mined with [$^3$H]7-OH-DPAT using a fixed concentration (±2.3 nM) of the G-protein mix, which corresponds to a G-protein/receptor ratio as indicated in Fig. 3. To compare the agonist binding properties of $hD_2$ and $hD_3$ receptors, we have prepared membranes from *E. coli* cells and incubated them with the G-protein mix. The apparent affinity of [$^3$H]7-OH-DPAT for $hD_3$ receptors in prepared membranes was virtually equal to that in $hD_3$R-*E. coli* cells, indicating that preparation of the membranes does not alter the binding properties of the receptor. [$^3$H]7-OH-DPAT had an apparent binding affinity of 1.7 ± 0.2 nM ($n = 4$) for untreated ($B_{max}$ = 2000 ± 300 fmol/mg of protein) and 1.1 ± 0.2 nM ($n = 4$) for G-protein-treated $hD_3$R-*E. coli* membranes ($B_{max}$ = 2200 ± 300 fmol/mg of protein); addition of GppNHp along with the G-protein mix, resulted in an apparent binding affinity of 2.0 ± 0.3 nM ($n = 4$) ($B_{max}$ = 2200 ± 200 fmol/mg of protein). As mentioned before, [$^3$H]7-OH-DPAT binding at $hD_2$R-*E. coli* membranes could not be determined in the absence of G-proteins. However, when the G-protein mix was added to the membranes, [$^3$H]7-OH-DPAT binding was clearly detectable at $hD_{2S}$R-*E. coli* membranes (see Fig. 2) with an apparent binding affinity of 3.5 ± 0.1 nM ($n = 2$) ($B_{max}$ = 160 ± 30 fmol/mg of protein); addition of GppNHp thereupon abolished [$^3$H]7-OH-DPAT binding. At $hD_{2L}$R-*E. coli* membranes, [$^3$H]7-OH-DPAT binding became apparent on addition of G-proteins, but the signal-to-noise ratio was too low for further characterization.

To determine the affinity of the G-protein mixture for $hD_{2S}$ and $hD_3$ receptors, we added increasing amounts of the G-protein mixture to $hD_{2S}$R- or $hD_3$R-*E. coli* membranes and measured the increase in [$^3$H]7-OH-DPAT binding, which was used at a concentration slightly lower than its determined apparent binding affinity in CHO cell membranes (i.e., 0.75 nM for $hD_3$ receptors and 2 nM for $hD_{2S}$ receptors). Addition of $G_{i/o}$-proteins increased [$^3$H]7-OH-DPAT binding to $hD_{2S}$R and $hD_3$R-*E. coli* membranes in a concentration-dependent way (Fig. 3). The slopes of these curves were near unity. Derived $pEC_{50}$ values were 8.35 ± 0.06 ($n = 4$) for $hD_3$R-*E. coli* and 8.12 ± 0.06 ($n = 4$) for $hD_{2S}$R-*E. coli*. Apparently, the affinity of the $G_{i/o}$-protein mix for both re-

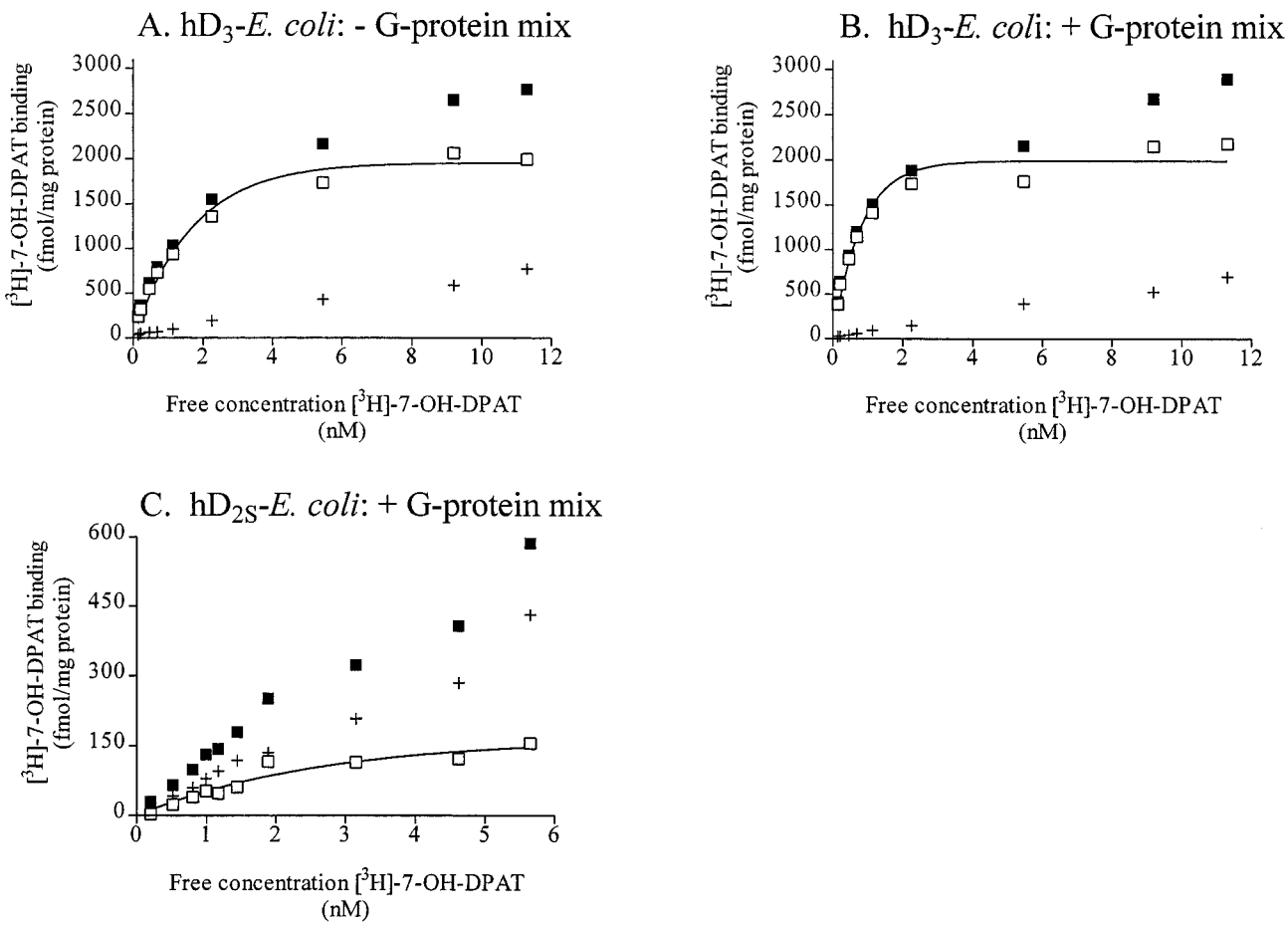


**Fig. 2.** Radioligand binding isotherms using [$^3$H]7-OH-DPAT in the absence or presence of a $G_{i/o}$-protein mix (2.3 nM) at $hD_3$-*E. coli* membranes (A) and in the presence of a $G_{i/o}$-protein mix (2.3 nM) at $hD_{2S}$-*E. coli* membranes (B). No specific [$^3$H]7-OH-DPAT binding to $hD_{2S}$-*E. coli* membranes could be detected in the absence of G-proteins. Experiments are performed as described under *Experimental Procedures*. Representative curves are shown in which each point was determined in duplicate. [$^3$H]7-OH-DPAT had an apparent binding affinity of 1.7 ± 0.2 nM ($n$ = 4) for untreated ($B_{max}$ = 2000 ± 300 fmol/mg of protein) and 1.1 ± 0.2 nM ($n$ = 4) for G-protein-treated $hD_3$R-*E. coli* membranes ($B_{max}$ = 2200 ± 300 fmol/mg of protein). [$^3$H]7-OH-DPAT had an apparent binding affinity of 3.5 ± 0.1 nM ($n$ = 2) ($B_{max}$ = 160 ± 30 fmol/mg of protein) for G-protein-treated $hD_{2S}$R-*E. coli* membranes.

ceptors was quite similar for $hD_3$ than for $hD_{2S}$ receptors (Student's $t$ test, $P < .05$). Indeed, assuming a bimolecular interaction between the receptor and the heterotrimeric G-protein, we estimated apparent dissociation binding constants of $1.4 \times 10^{-7}$ M in $hD_3$R-*E. coli* membranes and $9.4 \times 10^{-8}$ M in $hD_{2S}$R-*E. coli* membranes (for details of calculation see Fig. 3). Addition of the $G_{i/o}$-protein mix to $hD_{2S}$R-*E. coli* or $hD_3$R-*E. coli* membranes had no effect on antagonist binding (results not shown). At the highest concentration of the $G_{i/o}$-protein mix, we estimated a G-protein/receptor ratio of 260 for $hD_3$ and of 780 for $hD_{2S}$ receptors.

## Discussion

We have used an *E. coli* and CHO expression system to investigate the ligand binding properties of $hD_{2S}$, $hD_{2L}$, and $hD_3$ receptors in the absence or presence of G-proteins, respectively. The goal was to obtain information on their receptor-G-protein interaction.

**Successful Expression of $hD_{2S}$, $hD_{2L}$, and $hD_3$ Receptors in *E. coli*.** We have demonstrated expression of $hD_{2S}$, $hD_{2L}$, and $hD_3$ receptors in *E. coli* membranes. The antagonist [$^{125}$I]iodosulpride and partial agonist [$^3$H]7-OH-DPAT bound with similar affinity to $hD_3$ receptors expressed in *E. coli* and CHO cells (Table 1). In general, the apparent binding affinities and the rank order of potency of the antagonists and agonists tested were similar in $hD_3$R-*E. coli* cells and $hD_3$R-CHO cell membranes (Table 2). [$^3$H]Spiperone bound to $hD_{2S}$R-*E. coli* and $hD_{2L}$R-*E. coli* cell membranes with an apparent affinity comparable with $hD_{2S}$R-CHO and $hD_{2L}$R-CHO cell membranes (Table 1). The tested antagonists revealed apparent affinities at $hD_{2S}$R-*E. coli* and $hD_{2L}$R-*E. coli* membranes that were lower than their apparent affinities in $hD_{2S}$R-CHO and $hD_{2L}$R-CHO cell membranes, but the rank order of potency was similar, suggesting successful expression (Table 3). The lower apparent affinities of the ligands at $hD_2$ receptors in *E. coli* membranes may be attributed to sticking of the compounds and/or [$^3$H]spiperone (see Fig. 1, B and C: high nonspecific binding of [$^3$H]spiperone) to *E. coli* membranes. Alternatively, it may be attributed to the different composition of bacterial membranes; lower affinities of antagonists have also been observed for dopamine $D_2$ receptors expressed in *Saccharomyces cerevisiae* (Sander et al., 1994). Also, the different protein amount and assay volume in the binding assays for $hD_2$R-*E. coli* and -CHO membranes may lead to the lower apparent affinities of the compounds in *E. coli* membranes versus CHO membranes. Inhibition bind-

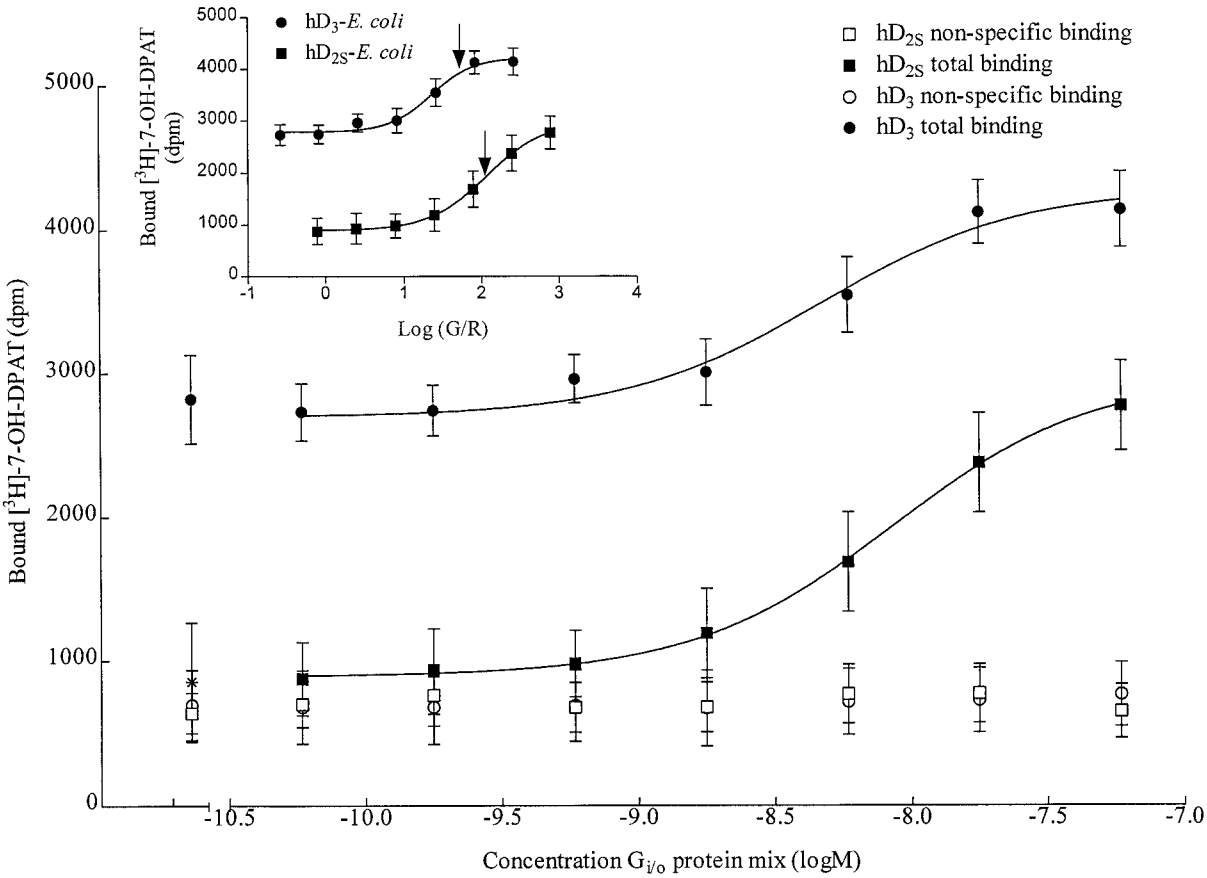


**Fig. 3.** Reconstitution of high affinity [$^3$H]7-OH-DPAT binding at $hD_{2S}$ and $hD_3$ receptors in *E. coli* membranes using a $G_{i/o}$-protein mix. Experiments are performed as described under *Experimental Procedures*. Curves represent the mean of four experiments ± S.D. Derived $pEC_{50}$ values are 8.35 ± 0.06 for $hD_3$R-*E. coli* and 8.12 ± 0.06 for $hD_{2S}$R-*E. coli*. The same amount of protein (35 $\mu$g) and incubation volume (0.5 ml) was used for $hD_{2S}$R-*E. coli* and $hD_3$R-*E. coli* membranes. The inset represents the increase of total bound [$^3$H]7-OH-DPAT as a function of the ratio of G-protein/receptor. The arrows indicate the ratio G-protein/receptor that corresponds to what was used for generating the [$^3$H]7-OH-DPAT saturation binding isotherms (see Fig. 2). Binding equilibrium constants were calculated assuming expression levels ($B_{max}$) of 2000 fmol/mg for $hD_3$ receptors and 1000 fmol/mg for $hD_{2S}$ receptors and with the equation: $K = (B_{max} - [D_xR{\cdot}G])[G]/[D_xR{\cdot}G]$ in which $[D_xR{\cdot}G]$ is the concentration of the high affinity dopamine receptor-G-protein complex (difference between binding in untreated and G-protein-mixed membranes) and [G] is the concentration of the exogenously added G-protein mix. $K_d$ values were calculated using the estimated concentrations of the reaction components at the level of the $pEC_{50}$ values of the G-protein mix. We found for the $hD_3R{\cdot}G_{i/o}$-protein an apparent $K_d$ of $1.4 \times 10^{-7}$ M and for the $hD_{2S}R{\cdot}G_{i/o}$-protein an apparent $K_d$ of $9.4 \times 10^{-8}$ M.

ing constants using [$^3$H]7-OH-DPAT in membranes of $hD_{2S}$R- or $hD_{2L}$R-expressing *E. coli* could not be determined due to the absence of radioligand binding at nanomolar concentration. In CHO and *E. coli* cell membranes, compounds had similar affinities for $hD_{2S}$ and $hD_{2L}$ receptors, respectively. This confirms previous findings and expands the idea of similar binding properties of these splice variants also to the G-protein-free environment in *E. coli* membranes (Schotte et al., 1996).

**G-Protein Uncoupling Decreases High Affinity Agonist Binding at $hD_2$ Receptors.** GppNHp treatment significantly decreased the affinity of [$^3$H]7-OH-DPAT at $hD_{2S}$R-CHO and $hD_{2L}$R-CHO cell membranes (Table 1). Moreover, the lack of [$^3$H]7-OH-DPAT binding at nanomolar concentration in $hD_{2S}$R-*E. coli* and $hD_{2L}$R-*E. coli* membranes indicates that $hD_2$ receptors have a low affinity for [$^3$H]7-OH-DPAT in the total absence of G-proteins. Probably, G-proteins in CHO membranes cannot be fully dissociated from their receptor by GppNHp leading to an intermediate ("low") affinity for agonists. It was observed that [$^3$H]7-OH-DPAT bound to $hD_{2S}$R-*E. coli* membranes in the presence of a $G_{i/o}$-protein mix with an affinity similar to what was found in $hD_{2S}$R-CHO cell membranes, indicating proper folding of the receptor and reconstitution of the high affinity state. This reconstitution was reversible, because GppNHp treatment abolished [$^3$H]7-OH-DPAT binding. Furthermore, we found significantly lower potencies (10–20-fold lower) of dopamine and 7-OH-DPAT for inhibition of [$^3$H]spiperone binding to $hD_{2S}$R- and $hD_{2L}$R-*E. coli* membranes (Table 3), indicating again that high affinity agonist binding to $D_2$ receptors requires the presence of G-proteins.

**G-Protein-Coupled and Uncoupled States of $hD_3$ Receptors Are Similar.** It is generally assumed that $D_3$ receptors poorly couple to G-proteins or that the investigated cell systems do not possess the suitable G-proteins to explain a small (or no) shift in high affinity agonist binding on GTP$\gamma$S treatment. The latter assumption is further substantiated by studies reporting the inability of $D_3$ receptors to regulate effector systems (Freedman et al., 1994; Tang et al., 1994). However, in recent publications it was shown that $D_3$ receptors do activate G-proteins (Vanhauwe et al., 1999). However, it has not been established whether the slight modulation of agonist binding by guanine nucleotides is due to a particular interaction between the $D_3$ receptor and its target G-protein or that it is due to specific properties of the receptor itself. Our results confirm that GppNHp treatment does

not decrease the affinity of an agonist ([$^3$H]7-OH-DPAT) for $hD_3$ receptors in CHO cell membranes. Most interestingly, we found that [$^3$H]7-OH-DPAT had similar affinities for $hD_3$ receptors in *E. coli* and CHO cell membranes (Table 1). This clearly shows that high affinity agonist binding to $hD_3$ receptor is almost independent of G-proteins, which makes it unlikely that a tight association between receptor and G-protein would prevent the effect of guanine nucleotides.

**Inverse Agonists Do Not Have Higher Affinities for Uncoupled $hD_2$ and $hD_3$ Receptors.** It was hypothesized that inverse agonists would have higher affinities for uncoupled than for coupled receptors (Costa et al., 1992). Antagonists tested in this study have been reported to be inverse agonists at $hD_{2S}$ (haloperidol, domperidone, risperidone, spiperone, and nemonapride) and $hD_3$ receptors (haloperidol and raclopride) (Griffon et al., 1996; Hall and Strange, 1997; Kozell and Neve, 1997; Malmberg et al., 1998; Vanhauwe et al., 2000). However, Malmberg et al. (1998) reported no shift to higher affinities for inverse agonists at $hD_3$ receptors after pertussis toxin treatment of the membranes. Here, we demonstrate that in *E. coli* membranes, which are devoid of G-proteins, these presumed inverse agonists do not have higher affinities for $hD_{2S}$, $hD_{2L}$, or $hD_3$ receptors than in CHO cell membranes. Therefore, the theory of the allosteric ternary complex model with regard to inverse agonists seems not to be generally applicable.

**A Purified Bovine G-Protein Mix Has a Comparable Affinity for $hD_3$ and $hD_2$ Receptors.** It is not known why $D_3$ receptors activate G-proteins (much) less efficiently than $D_2$ receptors. Several hypotheses have been put forward, such as inability to activate and/or couple to G-proteins, unavailability of suitable G-proteins, or a low affinity toward G-proteins. To address this issue, we have tried to estimate the affinity of a subset of pertussis toxin-sensitive G-proteins for the $hD_{2S}$ and $hD_3$ receptors. Therefore, we have reconstituted high affinity [$^3$H]7-OH-DPAT binding at $hD_{2S}$R- and $hD_3$R-*E. coli* membranes by addition of increasing concentrations of exogenous $G_{i/o}$-proteins, purified from bovine brain. As was found before, no specific [$^3$H]7-OH-DPAT binding was detectable in the absence of G-proteins at $hD_{2S}$R-*E. coli* membranes, in contrast to $hD_3$R-*E. coli* membranes. However, the G-protein mix increased [$^3$H]7-OH-DPAT binding in a dose-dependent manner at either receptor, whereas it had no effect on antagonist binding (latter results not shown).

Most surprisingly, the G-protein mix was at least as potent in increasing high affinity agonist binding at $hD_3$ receptors than at $hD_{2S}$ receptors. The estimated $K_d$ of $1.4 \times 10^{-7}$ M for the $hD_3R{\cdot}G_{i/o}$ complex and the $K_d$ of $9.4 \times 10^{-8}$ M for the $hD_{2S}R{\cdot}G_{i/o}$ complex corresponded to reported affinities of G-proteins for G-protein-coupled receptors. In addition, the G-protein/receptor ratio for half-maximal effect was similar to previous findings for $A_1$-adenosine and $5HT_{1A}$ receptors in *E. coli* membranes (Bertin et al., 1992; Jockers et al., 1994) and slightly higher for dopamine $D_{2S}$ receptors in Sf9 membranes (Grünewald et al., 1996). The G-protein/receptor ratio is probably overestimated due to the presence of inactive, purified G-protein contaminations and/or nonoptimal insertion of G-proteins in *E. coli* membranes. It cannot be concluded that more G-proteins per receptor were required for reconstituting high affinity binding at $hD_3$ receptors (versus $hD_{2S}$ receptors), because more receptors were expressed in the $hD_3$-*E. coli* membranes. Therefore, a more efficient way was to calculate the $K_d$ values based on our results (see Fig. 3 and legend), which showed that there was no difference in affinity between $hD_{2S}$ and $hD_3$ receptors for the $G_{i/o}$-protein mix. Until recently, it was generally believed that $hD_3$ receptors couple poorly to G-proteins when compared with $D_2$ receptors. Now, we found an equally strong interaction of $hD_3$ receptors compared with $hD_2$ receptors with a $G_{i/o}$-protein mix. Robinson and Caron (1996) suggested a weak interaction between $hD_3$ receptors and G-proteins due to a constrained receptor structure. We propose a strong interaction between $hD_3$ receptors and G-proteins, but a rigid receptor structure that is little influenced by G-protein coupling. The rigid $hD_3$ receptor activates G-proteins less efficient than $D_2$ receptors. Hence, the receptor-G-protein interaction can be described by two independent parameters, analogous to the agonist-receptor interaction, i.e., affinity and intrinsic activity. In this respect, $hD_3$ receptors have a high affinity and a low intrinsic activity at G-proteins, whereas $hD_2$ receptors have a high affinity and a high intrinsic activity at G-proteins. In future studies, it will be interesting to define the different regions of the receptor that contribute to either G-protein coupling or activation.

In conclusion, we have expressed in *E. coli* $hD_{2S}$, $hD_{2L}$, and $hD_3$ receptors, which retained their antagonist binding properties. In *E. coli*, as well as in CHO cell membranes, agonist binding to uncoupled receptors was severely impaired at $hD_{2S}$ and $hD_{2L}$ receptors, whereas only a small effect was observed at $hD_3$ receptors. We found that the low and high affinity states of $hD_3$ receptors are similar, whereas for $hD_{2S}$ and $hD_{2L}$ receptors they are clearly distinguishable. Furthermore, our results indicate that $hD_{2S}$ and $hD_3$ receptors have a similar affinity for an exogenously added $G_{i/o}$-protein mix. Thus, the reported low signaling capacity of $hD_3$ receptors, as compared with $hD_2$ receptors, is not due to a low affinity for G-proteins but may be attributed to a constrained structure of the receptor.

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