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Functional coupling of the human dopamine D_2 receptor with $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_o$ G proteins: evidence for agonist regulation of G protein selectivity

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1 The human dopamine D_{2long} (D_{2L}) receptor was expressed with four different G proteins in Sf9 cells using the baculovirus expression system. When co-expressed with G_i/G_o G proteins ($G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, or $G_o\alpha$, plus $G\beta_1$ and $G\gamma_2$), the receptor displayed a high-affinity binding site for the agonists (dopamine and NPA), which was sensitive to GTP (100 μM), demonstrating interaction between the receptor and the different G proteins.

2 The receptor to G protein ratio (R : G ratio) was evaluated using [^{3}H]-spiperone saturation binding (R) and [^{35}S]-GTPγS saturation binding (G). R : G ratios of 1 : 12, 1 : 3, 1 : 14 and 1 : 5 were found for G_{i1}, G_{i2}, G_{i3}, and G_o preparations, respectively. However, when R : G ratios of 1 : 2 and 1 : 12 were compared for G_{i2} and G_o, no difference was found for the stimulation of [^{35}S]-GTPγS binding.

3 Several agonists were tested for their ability to stimulate [^{35}S]-GTPγS binding to membranes co-expressing the receptor and various G proteins. All the compounds tested showed agonist activity in preparations expressing G_{i3} and G_o. However, for G_{i2} and G_{i1} preparations, compounds such as *S*-(−)-3-PPP and *p*-tyramine were unable to stimulate [^{35}S]-GTPγS binding.

4 Most of the compounds showed higher relative efficacies (compared to dopamine) and higher potencies in the preparation expressing G_o. Comparison of the effects of different agonists in the different preparations showed that each agonist differentially activates the four G proteins.

5 We conclude that the degree of selectivity of G protein activation by the D_{2L} receptor can depend on the conformation of the receptor stabilised by an agonist.

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Abbreviations: D_2, dopamine D_2 receptor; $G_{i/o}$, heterotrimeric GTP binding protein type i or o; GPCR, G protein-coupled receptor; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; m.o.i., multiplicity of infection; PCR, polymerase chain reaction; R_h, receptor higher affinity binding site; 3-PPP, 3-(3-hydroxyphenyl)-N-propylpiperidine

Introduction

The mechanisms of activation of G protein-coupled receptors (GPCRs) by agonists are still poorly understood, despite extensive work in the field. Several models have been developed to explain the mechanisms of GPCR activation, including the ternary complex model and its extensions (for a historical review, see Kenakin (1997). The extended ternary complex model proposes that the GPCRs exist in an inactive ground state (R), which can isomerise to a partially activated state (R*) that couples more efficiently to the G protein to form the active species (R*G). The formation of R*G may occur spontaneously, but agonists stabilise the active (R*) and (R*G) states. The model also proposes that GPCRs can activate G proteins, even in the absence of ligand ('constitutive activity') and that inverse agonists suppress this constitutive activity (Lefkowitz *et al*., 1993; Gether & Kobilka, 1998). There are, however, several experimental results which cannot be explained by the extended ternary complex model. For example, it was shown that the efficacy and potency of β_2-adrenergic receptor agonists depend on the specific purine nucleotide present for G protein activation and the specific G protein to which the β_2-adrenergic receptor couples (Seifert *et al*., 1999; Wenzel-Seifert & Seifert, 2000). In addition, some ligands have been shown to act as agonists in one setting and as inverse agonists in another setting (Chidiac *et al*., 1994). These findings and others (Zuscik *et al*., 1998; Thomas *et al*., 2000) suggest a multistate model of GPCR activation, in which ligands stabilise unique and ligand-specific GPCR conformations, which enable GPCRs to activate cognate G protein(s) in a ligand-specific manner (Gether & Kobilka, 1998; Kenakin, 2002).

The D_2 dopamine receptor, a member of the dopamine receptor family (which comprises five receptors, D_1–D_5), represents a good model for the analysis of the mechanisms of GPCR activation. Indeed, it has been shown to activate a large diversity of second messenger pathways, including inhibition of adenylyl cyclase (Hall & Strange, 1999), stimulation of phospholipase C (Vallar *et al*., 1990), potentiation of arachidonic acid release (Kanterman *et al*., 1991), regulation of K^+- and Ca^{2+}-channels activity (Lledo *et al*., 1992; Seabrook *et al*., 1994), as well

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as modulation of the activity of the Na^+/H^+ exchanger (Coldwell *et al*., 1999). Most of these signalling pathways involve interaction of D_2 receptors with G proteins of the $G_{i/o}$ family.

Alternative splicing of the D_2 receptor mRNA results in two isoforms of the receptor, termed D_{2S} (short) and D_{2L} (long) (Giros *et al*., 1989). The long isoform of the receptor differs from the short one by an additional 29 amino acid insert in the putative third intracellular loop. Both D_{2S} and D_{2L} are expressed widely in the brain and exhibit small differences in their pharmacological profile (Castro & Strange, 1993; Malmberg *et al*., 1993). The D_{2S} and D_{2L} have been shown to couple differently to G proteins (Montmayeur *et al*., 1993; Senogles, 1994). Interestingly, the D_{2S} receptor couples preferentially to G_{i1} over G_{i2} when expressed in Sf9 cells (Grünewald *et al*., 1996) and distinct roles for G protein subunits in the modulation of cAMP accumulation and calcium mobilisation by D_2 receptors were described (Liu *et al*., 1994; Ghahremani *et al*., 1999). It was also suggested that the 29 amino acid insert of D_{2L} might confer selectivity for interaction with G_{i2} (Guiramand *et al*., 1995). These observations suggest differences in the molecular mechanisms of the interaction of each isoform of the D_2 receptor with different G proteins, which may account for different signalling and therefore different functions. The differential coupling of the D_2 receptor to different G proteins may have pharmacological consequences. For example the relative efficacies of quinpirole and (+)-3-PPP are reversed when tested on D_2 receptors in the striatum and in the pituitary gland (Meller *et al*., 1992). This could reflect the interaction of the receptor with different G proteins in the two tissues.

We previously reported a selective interaction of the rat D_{2L} receptor with G_o over G_{i2} G proteins when expressed in Sf21 insect cells (Cordeaux *et al*., 2001). The interaction of D_{2L} with G_o appeared to be stronger than with G_{i2}. In addition, functional coupling of D_{2L} was more efficient at G_o than at G_{i2}. Moreover, the degree of selectivity depended on the agonist used. These findings prompted us to investigate the interaction of the D_{2L} receptor with other members of the $G_{i/o}$ family of G proteins using the same system. In order to compare the interaction of the D_{2L} receptor with the different G proteins in the same environment, we have reconstituted the interaction of the human D_{2L} receptor interaction with G_{i1}, G_{i2}, G_{i3}, and G_o G proteins by co-expression in baculovirus-infected Sf9 cells.

Materials

Bromocriptine, (+)-butaclamol, clozapine, dopamine, haloperidol, (−)-quinpirole, *m*-tyramine, *p*-tyramine, *S*-(−)-3-PPP, *R*-(+)-3-PPP, *R*-(−)-propylnorapomorphine (NPA) were purchased from Sigma (Saint Louis, Missouri, U.S.A.). Antibodies specific for different G protein subunits were from Chemicon International (Harrow, U.K.) and Santa Cruz Biotechnology (Calne, U.K.) as indicated. $[^3H]$-spiperone (15–30 Ci $mmol^{-1}$) and $[^{35}S]$-GTPγS (1099–1148 Ci $mmol^{-1}$) were from Amersham International, Buckinghamshire, U.K. All the other reagents were obtained as indicated.

Methods

Construction of recombinant baculoviruses

cDNA encoding for human D_{2L} dopamine receptor was subcloned into the vector TOPO® (Invitrogen) between a *Nde*I site at the 5′ end of the insert and an *EcoR*I site at the 3′ end of the insert, to produce the recombinant plasmid TOPOD2L. In order to add an epitope tag to the receptor in its amino terminus, complementary synthetic oligonucleotides encoding an HIV epitope tag sequence (McKeating *et al*., 1993) were designed as follows: 5′-AGTACTAGTATCA-GAGGCAAGGTACAACATATG-3′ and 5′-CATATGTTG-TACCTTGCCTCTGATACTAGTACT-3′. This introduces a 3′ *Nde*I site to the tag sequence. These oligonucleotides were annealed, and digested subsequently with *Nde*I. TOPOD2L was digested with *EcoR*I and *Nde*I. The DNA fragment and the HIV tag were then ligated. The ligation mixture was subjected to PCR to selectively amplify tagged receptor whilst at the same time adding an *Xho*I site and a start codon to the 5′ end of the tag. For this purpose the following primers were used: 5′-TTGAATTCTCAGCAGTGGAG-GATC-3′ and 5′-TTCTCGAGGATGGATAGTACTAG-TATCAGAGGC-3′. Both PCR products were digested with *Xho*I and *EcoR*I and ligated into the plasmid pBlueBac4.5 (Invitrogen), to produce the recombinant plasmid pBBHD2L. The plasmid was then co-transfected with Bac-N-Blue® DNA (Invitrogen) in Sf9 cells and underwent recombination, to produce recombinant baculovirus. All the constructs were verified by DNA sequencing. The baculoviruses expressing the human G protein subunits α_{i1-3}, α_o, β_1 and γ_2 were generously donated by T. Kozasa (University of Illinois, Chicago, U.S.A.). These baculoviruses were subjected to PCR to verify their purity. All the viruses were purified using plaque purification and amplified by serial infection of Sf9 insect cells.

Cell culture

Sf9 cells were grown in suspension in TC-100 medium supplemented with 10% foetal calf serum (FCS) and 0.1% pluronic F-68®. The cells were maintained at a density of 0.5 to 2.5×10^6 cells ml^{-1} and were split every two to three days. For infections, cells were seeded at a density of 0.3 to 0.6×10^6 cells ml^{-1} and infected when they reached a density of about 1×10^6 cells ml^{-1}. Infections were carried out with various multiplicities of infection (m.o.i.) of baculoviruses. When the receptor was expressed alone, an m.o.i. of 5 was used. When the receptor was co-expressed with G proteins the m.o.i. used were 6:10:10:10 for $D_{2L}:G\alpha_{i/o}:G\beta_1:G\gamma_2$, respectively. Preliminary experiments showed these combinations to be optimal for G protein activation. In the preparations where only D_{2L}, $G\beta_1$ and $G\gamma_2$ were used, m.o.i. of 6, 10 and 10 were used for each baculovirus, respectively. Cells were harvested 48 h after infection.

Membrane preparation

Cells were collected by centrifugation ($1700\times g$, 10 min, 4°C) and resuspended in 15 ml of buffer (mM) (HEPES 20, $MgCl_2$ 6, EDTA 1, EGTA 1, pH 7.4). Cell suspensions were then homogenised using an Ultra Turrax at setting 4–

5 for 20 s. The homogenate was centrifuged at $1700 \times g$ for 10 min and the supernatant was collected and centrifuged at $48,000 \times g$ for 1 h at 4°C. The resulting pellet was resuspended in buffer and stored at −80°C in aliquots of 500 μl. The protein concentration was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Immunoblot analysis

Membrane protein (20–40 μg) was incubated in 10 μl electrophoresis loading buffer (30 mM Tris-HCl, pH 6.8, 5% glycerol, 10% β-mercaptoethanol, 0.4% SDS, 0.04% bromophenol blue) and denatured by heating at 90°C for 5 min. Denatured membrane proteins were then separated by SDS polyacrylamide gel electrophoresis on 10% acrylamide gels. Samples were transferred to nitrocellulose membranes using the Biorad semi dry transfer system. Nitrocellulose membranes were incubated for 1 h with 5% dried milk (w/v) in buffer (mM) (NaCl 137, KCl 3, 0.1% Tween 20, Tris-HCl 25, pH 7.5). Membranes were then incubated overnight at 4°C with single primary antibodies (mAb3073 anti-$G\alpha_o$, 1 μg ml^{-1} (Chemicon); C-10 anti-$G\alpha_{i\text{-}3}$ (a rabbit polyclonal antibody able to recognise $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits), 1 μg ml^{-1} (Santa Cruz, specificity verified by Marston & Strange, unpublished); mAb3077 anti-$G\alpha_{i2}$, 1 μg ml^{-1} (Chemicon); C-16 anti-$G\beta_1$, 0.4 μg ml^{-1} (Santa Cruz); A-16 anti-$G\gamma_2$, 0.4 μg ml^{-1} (Santa Cruz, specificity verified in Cordeaux *et al.*, 2001) in buffer containing 5% dried milk (w/v). Membranes were washed four times with buffer (15 min each) before incubation with secondary antibody (anti-mouse ($G\alpha_o$, $G\alpha_{i2}$) or anti-rabbit ($G\alpha_{i\text{-}3}$, $G\beta_1$, $G\gamma_2$)) immunoglobulin horseradish peroxidase conjugate (Sigma, 1 : 5000) for 1 h. After three washes with buffer (10 min each), membranes were exposed to equal volumes of Enhanced Chemiluminescence (ECL) detection reagents 1 and 2 (Amersham) and bands were visualised after exposure of the membranes to Hybond-ECL X-ray film (Amersham) for between 30 s to 3 min.

[^{35}S]-GTPγS binding measurements

Stimulation of [^{35}S]-GTPγS (1099-1148 Ci mmol^{-1}; Amersham International, Buckinghamshire, U.K.) binding was measured on membranes co-expressing human dopamine D_{2L} and different G protein subunits. Membrane protein (5 μg) was incubated with 1 μM GDP and various concentrations of dopamine D_2 receptor ligands in buffer (mM) (HEPES 20, NaCl 100, $MgCl_2$ 6, pH 7.4) in a final volume of 80 μl. Reactions were performed in triplicate and were initiated by the addition of membrane proteins. After 30 min of pre-incubation at 30°C, 20 μl of [^{35}S]-GTPγS (0.1 nM final concentration) was added and the incubation continued for a further 30 min. Reactions were terminated by rapid filtration through Whatman GF/C glass fibre filters using a 96-well plate Brandel cell harvester, followed with four washes of 1.5 ml of ice-cold PBS. Radioactivity was determined as described above.

For [^{35}S]-GTPγS saturation binding, the final reaction volume was 1 ml. A final concentration (0.1 nM) of [^{35}S]-GTPγS was incubated in triplicate with a range of concentrations (0–60 nM) of non-radioactive GTPγS. The reactions were initiated by the addition of membranes. After 30 min incubation at 30°C, the reactions were terminated and radioactivity determined as described above. For each [^{35}S]-GTPγS saturation binding experiment, membranes prepared from cells expressing only receptor and $G\beta_1$ and $G\gamma_2$ subunits were used as control. [^{35}S]-GTPγS binding in the preparations containing receptor and G protein α, β, γ subunits was then corrected for this background binding. Saturation binding curves were derived to estimate the total number of G proteins expressed. The amount of ligand bound to G protein ($BOUND_{TOT}$) was calculated by equation 1: $BOUND_{TOT} = [^{35}S]\text{-}GTP\gamma S_{BOUND} \times GTP\gamma S_{TOT}/[^{35}S]\text{-}GTP\gamma S_{CONC}$, where [^{35}S]-GTP$\gamma S_{BOUND}$ is the corrected ligand binding, [^{35}S]-GTPγS_{CONC} is the [^{35}S]-GTPγS concentration in the tubes (0.1 nM) and GTPγS_{TOT} is [^{35}S]-GTPγS_{CONC} plus GTPγS concentration.

Radioligand binding assay

[^{3}H]-spiperone (15–30 Ci mmol^{-1}, Amersham International, Buckinghamshire, U.K.) saturation binding experiments were performed in a final volume of 1 ml buffer (mM) (HEPES 20, $MgCl_2$ 6, EDTA 1, EGTA 1, pH 7.4) and 25 μg of membrane protein per tube. Eight different concentrations of radioligand were used, ranging from approximately 10 pM to 2 nM. The reaction was initiated by the addition of membrane proteins and incubated for 3 h at 25°C. Reactions were terminated by rapid filtration through Whatman GF/C glass fibre filters using a Brandel cell harvester followed with four washes of 3 ml of ice-cold PBS (mM) (NaCl 140, KCl 10, KH_2PO_4 1.5, Na_2HPO_4 8). Filter discs were soaked in 2 ml of Optiphase Hi-Safe 3 (Wallac) for at least 6 h before the radioactivity was determined by liquid scintillation spectrometry. Non-specific binding was defined in the presence of 3 μM (+)-butaclamol. Assays were performed in triplicate. For [^{3}H]-spiperone competition binding experiments, a range of concentrations of competing ligand was incubated with a fixed concentration of radioligand (typically 0.45 nM) and the reactions were started and terminated as described above.

Analysis of data

Data were analysed using the computer program GraphPad Prism (GraphPad Software Inc.). [^{3}H]-spiperone saturation binding experiments were fitted to a one-site model to define the B_{max} (receptor expression level) and K_D (dissociation constant for [^{3}H]-spiperone). Competition experiments were fitted to a two-site binding and a one-site binding models and the best fit was determined using an F-test. IC_{50} values of competitors were derived from this analysis and the K_i (inhibition constants) values were derived using the Cheng & Prusoff (1973) equation. For [^{35}S]-GTPγS binding, concentration-response curves for agonists were analysed by non linear least squares regression fit and EC_{50} and E_{max} (maximum effect) values were derived from this analysis. Results are given as mean $\pm$ s.e.mean of the indicated number of experiments.

Statistical comparisons were performed using Analysis of Variance (ANOVA), followed by Tukey post-hoc test, where appropriate. A value of $P < 0.05$ was considered significant.

Results

Expression of dopamine D_{2L} receptor and G protein subunits

In saturation binding experiments, [^{3}H]-spiperone was found to label a homogeneous and saturable population of specific binding sites in Sf9 cells expressing the human dopamine D_{2L} receptor with or without G protein subunits. The B_{max} and the K_D values were analysed using one-way ANOVA, and were not significantly different between the five preparations ($P > 0.05$). The B_{max} and the K_D values for [^{3}H]-spiperone are summarised in Table 1. The expression of G protein subunits was analysed by immunoblot, using antibodies directed against the different subunits. Figure 1 shows the results of immunoblots performed on membranes co-expressing the D_{2L} receptor and different combinations of G protein subunits. Bands corresponding to the size of each G protein subunit were identified. No band was detected with any of the antibodies when the receptor was expressed in the absence of exogenous G protein (lane 1 on Figure 1).

Analysis of receptor : G protein ratio

In order to assess the G protein expression levels in our system, we used a method which takes into account the relatively high level of guanine nucleotide binding sites in Sf9 cells (Grünewald et al., 1996). Hence, we infected Sf9 cells with baculoviruses for D_{2L} and $G\beta_1$ and $G\gamma_2$ subunits only (to switch off expression of endogenous proteins). G protein levels were determined using [^{35}S]-GTPγS saturation binding and the level of [^{35}S]-GTPγS binding in the receptor/G$\beta\gamma$ preparation was subtracted from that in the preparations containing the heterotrimers (Figure 2a, c, e and g). Data were then transformed into saturation curves as described in the Methods section and the apparent K_D for GTPγS as well as the relative G protein levels (B_{max}) were derived for the different preparations (Figure 2b, d, f and h) and are summarised in Table 1. The apparent K_D for GTPγS with different preparations was not significantly different between the preparations containing the four G proteins (one-way ANOVA, $P > 0.05$). Based on these data and the B_{max} values for [^{3}H]-spiperone binding, the R : G ratios in the different preparations were calculated and data are given in Tables 1 and 2.

Effects of dopamine and dopamine receptor agonists on [^{35}S]-GTPγS binding

When the receptor and G protein subunits were expressed using m.o.i. of 6/10/10/10 (R/α/β_1/γ_2) the R : G ratios in the different preparations were not equivalent (Table 1). Indeed the R : G ratios for the G_{i2} and G_o preparations were found to be lower than that for the G_{i1} and G_{i3} preparations. We therefore sought to analyse the effect of varying the R : G ratio on agonist activity at G_{i2} and G_o. Thus, by varying the m.o.i. of the baculoviruses used, two preparations (with R : G ratios of ~1 : 2 and ~1 : 12) were generated for each R/G combination. The effect of dopamine in preparations expressing G_{i2} and G_o with varying R : G ratios is summarised in Table 2. Thus, the maximal effect and the potency of dopamine were similar (one-way ANOVA, $P > 0.05$) for the two R : G ratios, for G_{i2} and G_o preparations (Table 2). We also tested the effect of other dopamine D_2 receptor agonists at the two R : G ratios. As

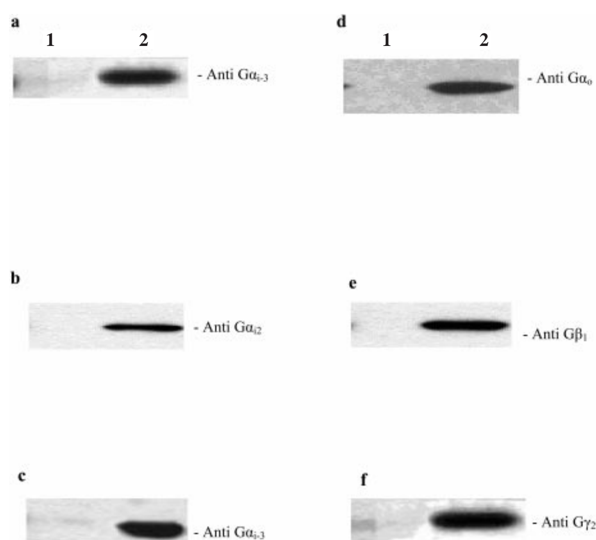


Figure 1 Expression of G protein subunits in Sf9 cells. Sf9 membranes expressing the D_{2L} receptor alone (lane 1) or co-expressing the D_{2L} receptor with different combinations of G protein subunits (lane 2) were separated by SDS–PAGE, transferred to nitrocellulose filters, and probed with the indicated antibodies as described in the Methods section. (a) $D_{2L}G_{i1}\beta_1\gamma_2$; (b) $D_{2L}G_{i2}\beta_1\gamma_2$; (c) $D_{2L}G_{i3}\beta_1\gamma_2$; (d) $D_{2L}G_o\beta_1\gamma_2$; (e) and (f) $D_{2L}G_{i/o}\beta_1\gamma_2$. Representative experiments performed on each membrane preparation are shown.

Table 1 Expression levels of human dopamine D_{2L} receptor (R) and G protein (G) in Sf9 cells

	[^{3}H]-spiperone saturation binding		[^{35}S]-GTPγS saturation binding		
Preparation	B_{max} (R)	$pK_D \pm$ s.e.mean (K_D, pM)	B_{max} (G)	$pK_D \pm$ s.e.mean (K_D, nM)	R : G ratio
D_{2L}	2.03±0.22	9.98±0.05 (105)			
$D_{2L}\alpha_{i1}\beta_1\gamma_2$	2.11±0.43	10.00±0.02 (100)	24.52±2.01	8.29±0.03 (5.13)	~1 : 12
$D_{2L}\alpha_{i2}\beta_1\gamma_2$	2.80±0.27	10.00±0.06 (100)	8.32±1.3	8.48±0.03 (3.31)	~1 : 3
$D_{2L}\alpha_{i3}\beta_1\gamma_2$	2.17±0.17	10.00±0.06 (100)	30.32±2.17	8.27±0.02 (5.37)	~1 : 14
$D_{2L}\alpha_0\beta_1\gamma_2$	2.88±0.16	10.00±0.07 (100)	15.06±2.47	8.40±0.02 (3.98)	~1 : 5

B_{max} values for the D_{2L} receptor (R) were determined using [^{3}H]-spiperone binding and for G proteins (G) using [^{35}S]-GTPγS saturation binding as described in the Methods section. Data are mean ± s.e.mean from 4–7 experiments (R) or 7–14 experiments (G). The B_{max} values are expressed in pmol mg^{-1} of protein.

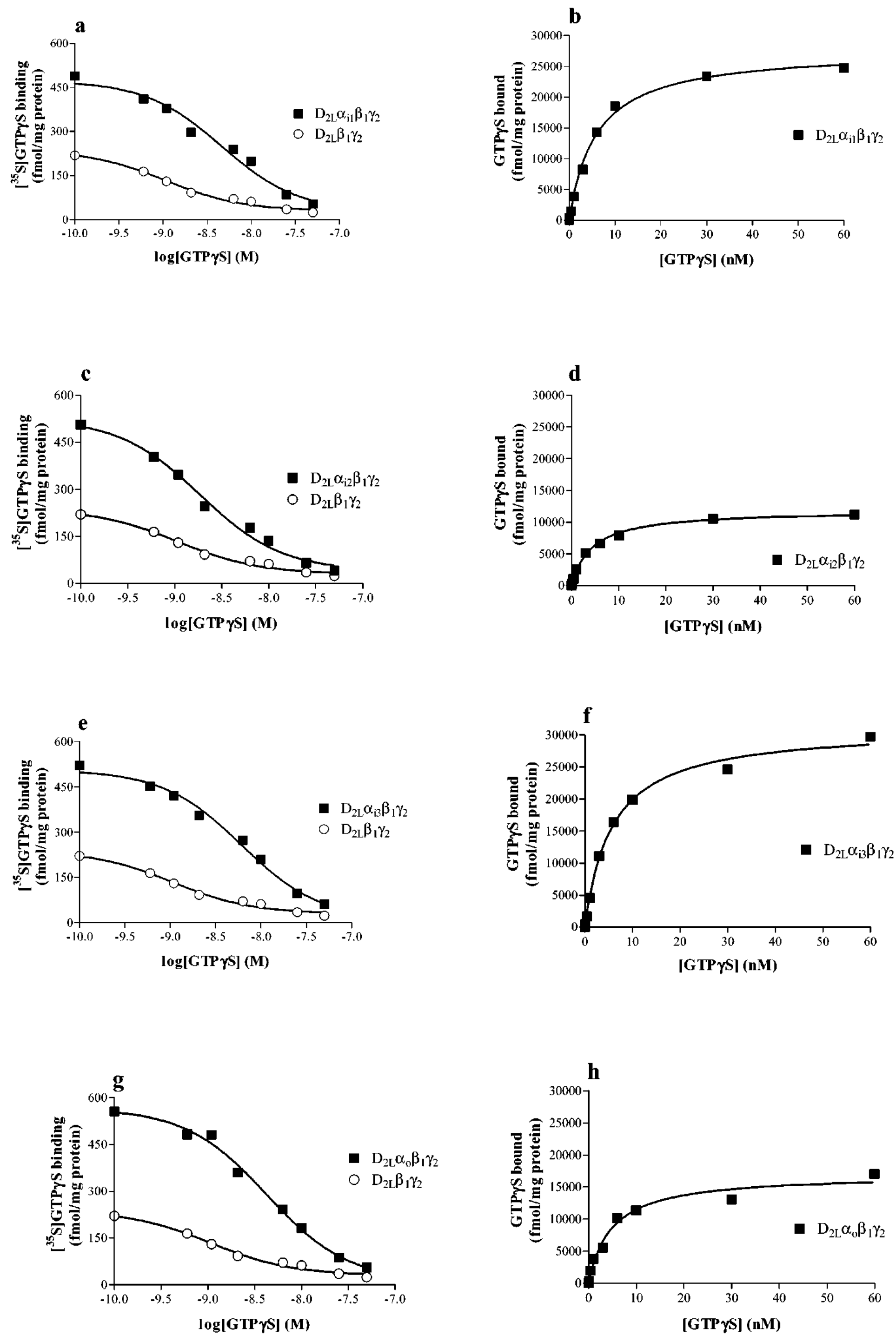


Figure 2 G protein levels analysed by [^{35}S]-GTPγS saturation binding. [^{35}S]-GTPγS saturation binding experiments were performed on Sf9 membranes expressing D_{2L} receptor and G_{i1} (a, b), G_{i2} (c, d), G_{i3} (e, f), and G_o (g, h), as described in the Methods section. Data are from representative experiments repeated as in Table 1.

shown in Figure 3, the relative efficacies, as well as the potencies of bromocriptine, NPA, and quinpirole were similar (one-way ANOVA, $P>0.05$) at the two R : G ratios. In addition, for the G_{i2} preparation, the effect of *S*-(−)-3PPP and *p*-tyramine were assessed at two R : G ratios. In one experiment out of five, *S*-(−)-3PPP showed a slight agonist effect (E_{max} 11% over basal level, equivalent to 14% the efficacy of dopamine). Thus, the two compounds were virtually inactive in the G_{i2} preparation, even when the R : G ratio was increased (Figure 3).

As the stimulation of [^{35}S]-GTPγS binding in these preparations seemed to be independent of the R : G ratio, we pooled data from the different preparations. The average maximal effect of dopamine for stimulation of [^{35}S]-GTPγS

Table 2 Dopamine stimulation of [^{35}S]-GTPγS binding to membranes expressing D_{2L} and G_{i2} or G_o

	$D_{2L}\alpha_{i2}\beta_1\gamma_2$		$D_{2L}\alpha_o\beta_1\gamma_2$	
	E_{max} (% basal)	$pEC_{50}\pm$s.e.mean (EC_{50}, nM)	E_{max} (% basal)	$pEC_{50}\pm$s.e.mean (EC_{50}, nM)
R:G∼1:2	96±11	5.41±0.11 (3900)	44±6	6.35±0.27 (450)
R:G∼1:12	98±14	5.56±0.16 (2800)	49±2	6.30±0.25 (500)

[^{35}S]-GTPγS binding experiments were conducted as described in the Methods section on preparations with different R:G ratios as indicated. The R:G ratios were determined as described in Table 1. Data shown are mean±s.e.mean from 7–10 experiments. The E_{max} and pEC_{50} values were not significantly different between the preparations with different R:G ratios (one-way ANOVA, $P>0.05$). Basal [^{35}S]-GTPγS binding levels in the preparations with different R:G ratios were significantly different (one-way ANOVA, $P<0.05$) only in the $D_{2L}\alpha_o\beta_1\gamma_2$ preparation, where basal binding was ∼30% higher in the R:G∼1:12 preparation (one-way ANOVA, $P=0.005$, data not shown).

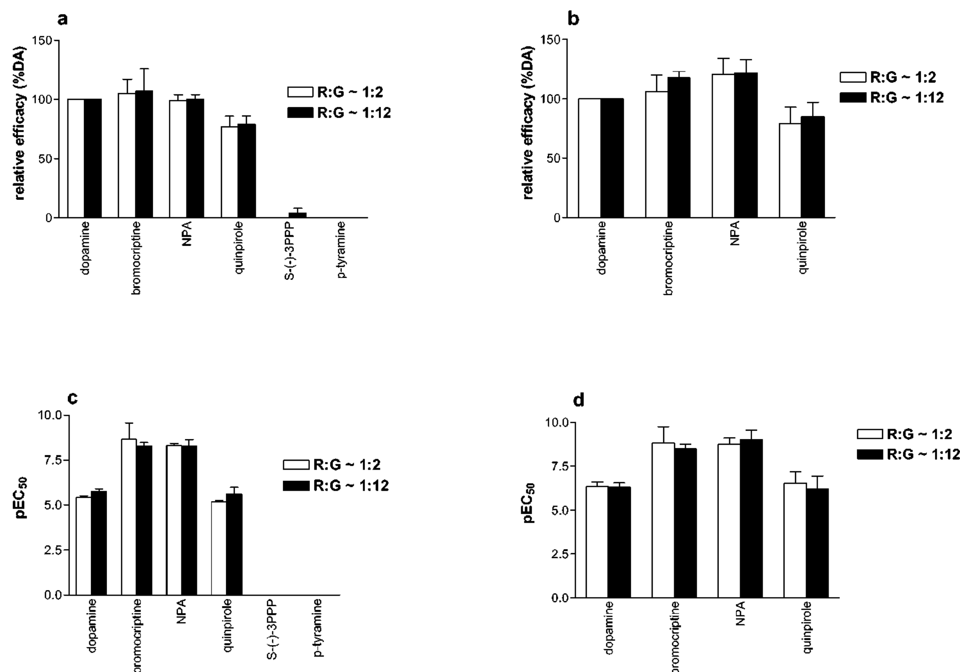


Figure 3 Stimulation of [^{35}S]-GTPγS binding by agonists in membranes of Sf9 cells expressing D_{2L} receptor and G_{i2} or G_o at various R:G ratios. The stimulation of [^{35}S]-GTPγS binding was determined as described in the Methods section on membranes expressing D_{2L} receptor and G_{i2} (a, c), or G_o (b, d). The R:G ratios were calculated as in Table 1. Data shown are mean±s.e.mean of 3–17 experiments.

binding in the different preparations was 44±4%, 94±14%, 80±9% and 47±3% over basal levels for preparations expressing D_{2L} and G_{i1}, G_{i2}, G_{i3}, and G_o, respectively. No agonist effect was found in Sf9 cells expressing the D_{2L} receptor in the absence of exogenous G protein (data not shown).

A series of dopamine agonists were then tested for their ability to stimulate [^{35}S]-GTPγS binding in the different preparations (Figure 4, Table 3). All of the agonists tested showed intrinsic activity at G_{i3} and G_o G proteins (Figure 4c and d). Compounds such as bromocriptine and NPA were potent agonists in all the four preparations. They also behaved as full agonists (relative to dopamine) in all preparations, with the exception of the preparation expressing G_{i3}, where bromocriptine was a partial agonist (Figure 4c, Table 3). All the other compounds tested showed partial agonist activities (relative to dopamine) in the preparations expressing G_{i3} and G_o. However, *S*-(−)-3-PPP was unable to stimulate [^{35}S]-GTPγS binding (Figure 4a and b) in the G_{i1} and G_{i2} preparations, while *p*-tyramine was unable to stimulate [^{35}S]-GTPγS binding in the G_{i2} preparation (Figure 4b). Compounds such as dopamine, NPA, quinpirole, and *m*-tyramine showed their highest potencies in the G_o preparation (Table 3). Bromocriptine had similar potency in each of the preparations.

Dopamine D_{2L} receptor and G protein interaction

Interaction of the dopamine D_{2L} receptor with G proteins was assessed in agonist competition experiments versus [^{3}H]-spiperone binding to membranes prepared from cells co-expressing the receptor and the different G protein subunits. Figure 5 shows competition binding between [^{3}H]-spiperone and dopamine or NPA. In the absence of GTP, competition curves for both compounds were best fitted to a two binding site model (F-test, $P<0.002$), with higher and lower affinity binding sites defined by dissociation constants K_h, and K_l. The percentage of higher affinity binding sites found with

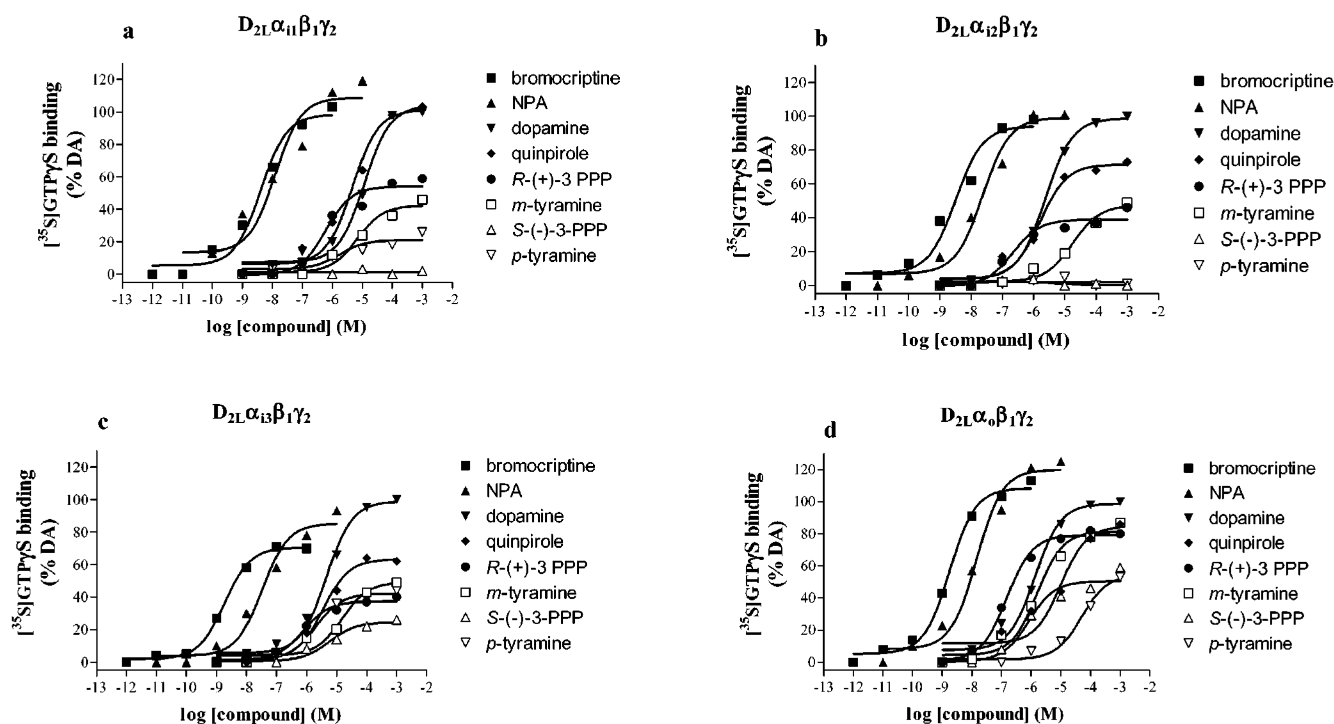


Figure 4 Stimulation of [^{35}S]-GTPγS binding by agonists in membranes of Sf9 cells expressing the D_{2L} receptor and various G proteins. The stimulation of [^{35}S]-GTPγS binding by agonists was determined as described in the Methods section on membranes expressing D_{2L} receptor and G_{i1} (a), G_{i2} (b), G_{i3} (c) or G_o (d). Data are representative stimulation curves replicated as in Table 3.

Table 3 Agonist stimulation of [^{35}S]-GTPγS binding to membranes expressing D_{2L} receptor and various G proteins

	$D_{2L}\alpha_{i1}\beta_1\gamma_2$		$D_{2L}\alpha_{i2}\beta_1\gamma_2$		$D_{2L}\alpha_{i3}\beta_1\gamma_2$		$D_{2L}\alpha_{o}\beta_1\gamma_2$	
	E_{max} (% DA)	pEC_{50}±s.e.mean (EC_{50}, nM)	E_{max} (% DA)	pEC_{50}±s.e.mean (EC_{50}, nM)	E_{max} (% DA)	pEC_{50}±s.e.mean (EC_{50}, nM)	E_{max} (% DA)	pEC_{50}±s.e.mean (EC_{50}, nM)
Dopamine	100	5.49±0.30 (3240)	100	5.52±0.13 (3000)	100	5.60±0.15 (2510)	100	6.32±0.18 (48)
NPA	117±15	8.12±0.37 (8)	99±2	8.29±0.15 (5)	103±8	7.73±0.23 (19)	125±8	8.90±0.27 (1.3)
Bromocriptine	112±13	8.50±0.17 (3)	99±14	8.48±0.56 (3)	76±6	8.86±0.07 (1.4)	112±7	8.64±0.21 (2.2)
Quinpirole	98±7	5.49±0.36 (3240)	78±8	5.38±0.48 (4200)	68±7	5.73±0.43 (1860)	82±10	6.58±0.46 (260)
m-tyramine	45±6	4.82±0.53 (15100)	57±16	4.88±0.10 (13200)	48±1	5.55±0.67 (2820)	85±4	6.08±0.44 (830)
p-tyramine	27±5	6.01±1.21 (1000)	na		46±8	4.32±0.75 (47900)	50±3	4.45±0.12 (35500)
R-(+)-3-PPP	58±6	5.72±0.80 (1900)	46±13	5.97±0.70 (1070)	49±6	5.48±0.61 (3310)	75±6	6.12±0.3 (760)
S-(−)-3-PPP	na		na		20±3	5.86±0.31 (1380)	64±8	5.65±0.34 (2240)

[^{35}S]-GTPγS binding experiments were conducted as described in the Methods section and the concentration-response curves analysed to provide the EC_{50} and the E_{max} (relative to the maximal dopamine effect). Data shown are mean±s.e.mean from 3–19 experiments. na = no activity.

each agonist was comparable (one-way ANOVA, $P>0.05$) between the four preparations (Table 4). As expected, the affinity of NPA was higher than that of dopamine in each of the preparations. In the presence of a high concentration of GTP (100 μM), the competition curves were best fitted to a one binding site model (F-test, $P<0.05$). The affinity of dopamine and NPA measured in the presence of GTP (pK_{GTP}) was similar (one-way ANOVA, $P>0.05$) to their lower affinity (pK_l) measured in the absence of GTP (Table 4).

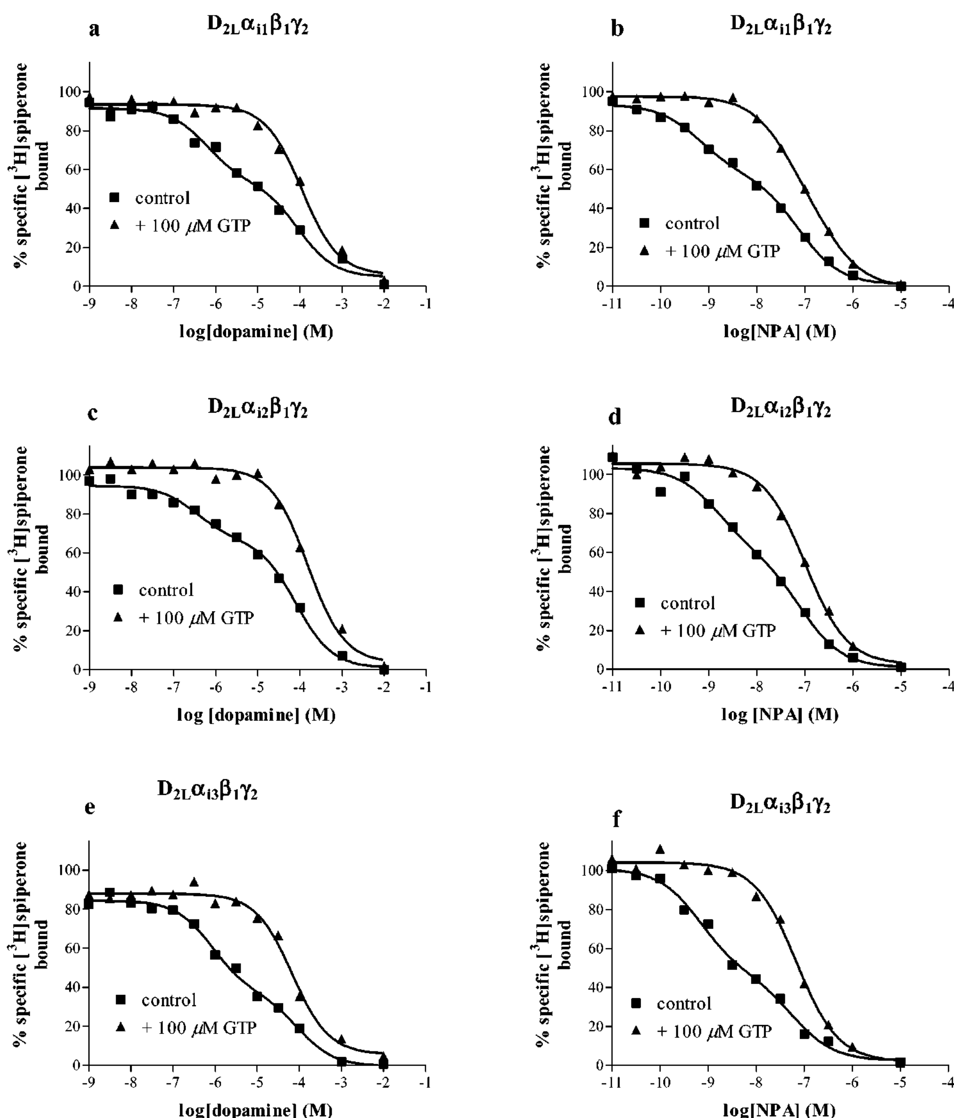
Competition of dopamine or NPA for [^{3}H]-spiperone binding also revealed a higher affinity binding state of the receptor when the D_{2L} receptor was expressed without exogenous G protein subunits (Figure 5i, j). This higher affinity binding site was sensitive to GTP (100 μM), with pK_{iGTP} values comparable (one-way ANOVA, $P>0.05$) to the pK_l values for both compounds (Figure 5, Table 4).

The binding of several antagonists was also tested in competition binding experiments versus [^{3}H]-spiperone. As shown in Figure 6, all the competition curves were best fitted by a one binding site model (F-test, $P<0.05$) and the rank order of affinity was (+)-butaclamol > haloperidol > clozapine ⩾ raclopride. The derived K_i values are consistent with those obtained for the receptor expressed in mammalian cell lines and they show that the dopamine D_{2L} receptor expressed in Sf9 cells behaves similarly to a system where the receptor couples exclusively with endogenous mammalian G proteins.

Discussion

In the present study, we have analysed the interaction of the D_2 dopamine receptor with G proteins by co-expressing human D_{2L} with G_{i1}, G_{i2}, G_{i3} and G_o in Sf9 cells using the baculovirus system. This study extends our previous observations on the rat D_{2L} receptor (Cordeaux *et al.*, 2001). Previous studies have shown that D_2 receptors can interact with and signal through pertussis toxin-sensitive G proteins ($G_{i/o}$) to couple to different pathways (see for example Montmayeur *et al.*, 1993; Senogles, 1994; Ghahremani *et al.*, 1999). These findings strongly suggested a differential coupling of the D_2 receptors with the different subtypes of G proteins. However, most of these interactions have been analysed in a system measuring intracellular events (i.e. cAMP accumulation, calcium mobilisation), where factors such as signal amplification may complicate interpretation. Furthermore, no study has analysed the four subtypes of G proteins in a well-defined system. Our present study is therefore the first to address the question of dopamine D_{2L} interaction with the four G proteins in a well-defined system using a method ([^{35}S]-GTPγS binding), which allows the observation of direct interaction between the receptor and the G proteins.

We have assessed the receptor (R) to G protein (G) ratio using [^{3}H]-spiperone and [^{35}S]-GTPγS saturation binding experiments to measure R and G levels, respectively. Others reported similar methods for determination of G protein levels (Traynor & Nahorski, 1995; Newman-Tancredi *et al.*, 2000). In the present study, we sought to use a method in which no GDP was present and we also take into account the endogenous G proteins or guanine nucleotide-binding proteins by using cells infected with D_{2L} and β_1/γ_2 only. We assumed that the binding of [^{35}S]-GTPγS to membranes expressing $D_{2L}\beta_1\gamma_2$ corresponds to either endogenous G proteins or other proteins as stated above. These values were, therefore, subtracted from those where receptor was expressed with heterotrimeric G protein (Figure 2). We obtained R : G ratios varying between 1 : 3 and 1 : 14, with the highest ratio being seen with G_{i3} preparations (Table 1). These ratios are somewhat lower than those obtained in other systems (see for example Wenzel-Seifert *et al.*, 1998; Neubig,



1994; Ransnäs & Insel, 1988). This variation may reflect differences in the methodology for determination of G protein levels or differences between expression systems.

The receptor to G protein ratio (R : G ratio) has been found to affect both the efficacy and the potency of ligands in several systems (see for example Pauwels *et al.*, 2000; Newman-Tancredi *et al.*, 2000). It was therefore necessary to analyse the effect of the R : G ratio on the signalling event. Thus, for the preparations expressing G_{i2} and G_o we found that both the efficacies and potencies of dopamine receptor agonists for the stimulation of [^{35}S]-GTPγS binding were similar (one-way ANOVA, $P>0.05$) for different R : G ratios (Table 2, Figure 3). It seems, therefore, that the overall R : G ratio in the cells may not reflect the functional ratio at a particular receptor. This is not unprecedented as in studies on the reconstitution of receptors and G proteins, even though an R : G ratio much less than one was used, the resulting preparation behaved as though the R : G ratio was greater than one (Jakubik *et al.*, 1998). It may be that receptors and G proteins enter micro domains in the cells with different R : G ratios and that these do not reflect the overall R : G ratio.

A range of agonists was tested for their ability to stimulate [^{35}S]-GTPγS binding to membranes expressing D_{2L} and the different G proteins. All the compounds tested showed agonist activity in the preparations expressing G_o and G_{i3}, whereas in the G_{i1} and G_{i2} preparations some agonists were

Table 4 Binding of dopamine and NPA to membranes of Sf9 cells expressing the D_{2L} dopamine receptor and different G proteins

	$D_{2L}\alpha_{i1}\beta_1\gamma_2$		$D_{2L}\alpha_{i2}\beta_1\gamma_2$		$D_{2L}\alpha_{i3}\beta_1\gamma_2$		$D_{2L}\alpha_o\beta_1\gamma_2$		D_{2L}	
	dopamine	*NPA*	*dopamine*	*NPA*	*dopamine*	*NPA*	*dopamine*	*NPA*	*dopamine*	*NPA*
% R_h	44±4	52±4	40±5	45±3	49±3	51±4	42±5	53±5	25±4	29±6
pK_h±s.e.mean	7.86±0.39	10.00±0.06	7.49±0.10	9.72±0.12	7.38±0.34	10.00±0.08	7.91±0.10	10.23±0.14	7.42±0.40	10.14±0.45
(K_h, nM)	(14)	(0.1)	(32)	(0.2)	(42)	(0.1)	(12)	(0.06)	(15)	(0.07)
pK_l±s.e.mean	5.10±0.40	8.04±0.12	5.06±0.07	8.03±0.08	4.75±0.24	8.11±0.13	5.26±0.16	8.10±0.14	4.71±0.01	7.98±0.06
(K_h, nM)	(7900)	(9)	(8700)	(9)	(17800)	(8)	(5500)	(8)	(19500)	(10)
pK_{GTP}±s.e.mean	4.64±0.34	7.75±0.09	4.75±0.08	7.86±0.05	4.72±0.16	7.82±0.06	4.88±0.06	7.94±0.06	4.70±0.07	7.80±0.04
(K_{GTP}, nM)	(22900)	(18)	(17800)	(14)	(19100)	(15)	(13200)	(11)	(20000)	(16)

Binding of dopamine and NPA was determined in competition experiments *vs* [^{3}H]-spiperone binding on membranes expressing D_{2L} receptor alone or co-expressing the D_{2L} receptor and various G proteins, as described in the Methods section. Competition curves were described best by two binding site models (F-test, $P<0.002$) in all cases in the absence of GTP and values for the dissociation constant at the higher (K_h) and lower (K_l) affinity sites and the percentage of higher affinity sites (% R_h) are given. In the presence of GTP (100 μM) all competition curves were fitted best by a one binding site model (F-test, $P<0.05$). Data are mean±s.e.mean from 4–6 experiments.

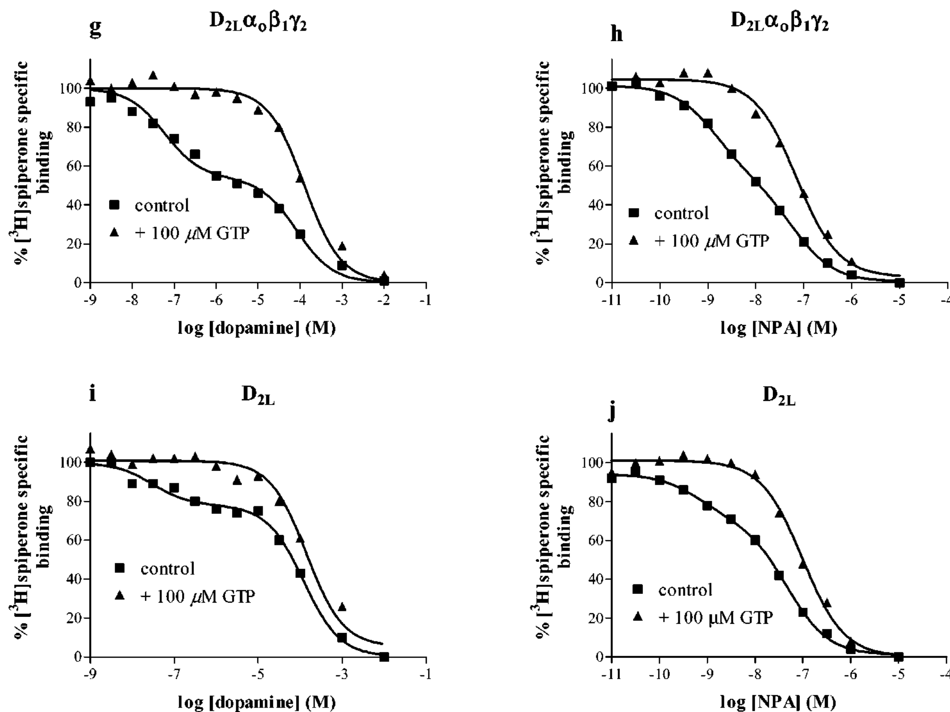


Figure 5 Agonist binding to membranes of Sf9 cells expressing the D_{2L} dopamine receptors and various G proteins. The binding of dopamine or NPA to the D_{2L} receptor co-expressed with G_{i1} (a, b), G_{i2} (c, d), G_{i3} (e, f), G_o (g, h) or alone (i, j) was determined in competition *vs* [^{3}H]-spiperone binding in the absence or presence of 100 μM GTP, as described in the Methods section. Data shown are representative experiments replicated as in Table 4 and the curves are the best fit curves to one site (+GTP) (F-test, $P<0.05$) or two site models (control) (F-test, $P<0.002$).

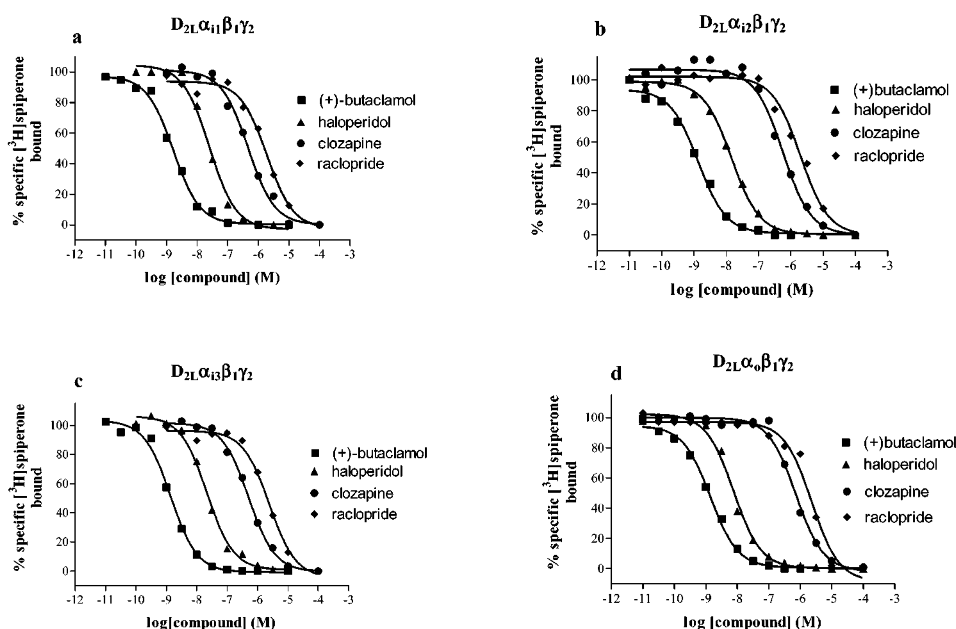


Figure 6 Antagonist binding to membranes of Sf9 cells expressing the D_{2L} receptor and various G proteins. The binding of (+)-butaclamol, haloperidol, clozapine, and raclopride to the D_{2L} receptor co-expressed with G_{i1} (a), G_{i2} (b), G_{i3} (c) or G_o (d) was determined in competition vs [^{3}H]-spiperone binding as described in the Methods section. Data shown are representative experiments replicated two to three times and the curves are the best fit curves to a one binding site model. Mean K_i values (nM) for the different compounds are as follows: (+)-butaclamol (0.25, 0.30, 0.33, 0.31); haloperidol (3.9, 2.4, 4.0, 1.8); clozapine (87, 93, 100, 115); raclopride (417, 372, 447, 240) in D_2G_{i1}, D_2G_{i2}, D_2G_{i3}, D_2G_o, respectively.

unable to stimulate [^{35}S]-GTPγS binding. Most of the compounds showed their highest efficacy (relative to that of dopamine) in the preparation expressing G_o (Table 3). This suggests a stronger or a more productive interaction between D_{2L} and G_o, and agrees with previous observations on the rat proteins (Cordeaux *et al.*, 2001). Nevertheless, for some compounds such as quinpirole, bromocriptine and NPA, the relative efficacies observed at G_{i1} were comparable to those seen at G_o. NPA and bromocriptine behaved as potent agonists at all the G proteins with two to three orders of magnitude higher potency compared to dopamine (Figure 4 and Table 3). The potency of several agonists (dopamine, NPA, quinpirole, *m*-tyramine, *R*-(+)-3-PPP) was slightly higher in the G_o preparation. However, bromocriptine had comparable potency in each preparation. Indeed the potency of bromocriptine in the present study was similar to that reported for the D_2 receptor expressed in other recombinant systems (Gardner *et al.*, 1996). It seems, therefore, that the potency of this compound is not dependent on the nature of the G protein and we have speculated that this may be because bromocriptine stabilises the receptor in a conformation close to that in the fully activated R*G state (Cordeaux *et al.*, 2001).

The monohydroxylated compounds *S*-(−)-3-PPP and *p*-tyramine were partial agonists in the G_o and G_{i3} preparations but not in the G_{i2} preparation. In the G_{i1} preparation, *S*-(−)-3-PPP exhibited partial agonist activity and *p*-tyramine was unable to stimulate [^{35}S]-GTPγS binding. These data suggest that the binding of these ligands to the receptor induces a conformation of the receptor which differentially couples to or activates G proteins, with weak interaction occurring between the receptor and G_{i1} and G_{i2} and more efficient interaction occurring with G_{i3} and G_o. Interestingly, both compounds appeared ineffective in the G_{i2} preparation, even at a higher R:G ratio. This supports the hypothesis of a weak interaction between the G_{i2} and the D_{2L} receptor occupied by these agonists. Others have reported increased efficacy of agonists for receptors signalling via G_o as compared to signalling via G_i proteins (Yang & Lanier, 1999; Francken *et al.*, 2000). Although the data of the present study show differences in the interaction of one receptor with different G proteins, they do not provide clear evidence for agonist-directed receptor trafficking as proposed by Kenakin (1995) and described in several studies (see for example Berg *et al.*, 1998; Robb *et al.*, 1994; Spengler *et al.*, 1993). In fact no clear reversal in the efficacy or the potency of the ligands was observed. Nevertheless, the present data imply that different agonists select different conformations of the receptor with different affinities for G proteins or abilities to activate them. Thus, the receptor/G protein combination dictates the precise pharmacological profile of the observed response.

The human dopamine D_{2L} receptor expressed in Sf9 cells, with or without exogenous G proteins, exhibited a K_D for [^{3}H]-spiperone similar to that of D_2 receptors expressed in mammalian cell lines (Gardner *et al.*, 1996) or native receptor (Withy *et al.*, 1981). Competition binding using the agonists dopamine and NPA revealed a high affinity binding component, which was sensitive to GTP, suggesting interaction of the receptor with co-expressed G proteins. The higher affinity agonist binding found in the preparations where D_{2L} was expressed alone may correspond to the interaction of the receptor with either endogenous G proteins or guanyl nucleotide-binding proteins that are not G proteins. In line with this, several studies have reported the presence of endogenous G proteins in Sf9 cells (Graber *et al.*, 1992;

Mulheron *et al*., 1994; Parker *et al*., 1991; Boundy *et al*., 1996). It is possible that in our system the D_{2L} receptor interacts with these endogenous proteins in the absence of exogenous G protein. This interaction does not however lead to activation, as we did not observe any agonist effect at the receptor expressed alone. In addition, immunoblot analysis did not reveal any endogenous G protein in our study (Figure 1). We therefore conclude that endogenous G proteins in Sf9 cells may interact with the human dopamine D_{2L} receptor but that this interaction does not lead to activation of G proteins.

In conclusion, this study shows a selective interaction of the D_{2L} receptor with G proteins when expressed in Sf9 cells. Our data suggest that agonists can regulate the interaction of dopamine D_{2L} receptor with G proteins, independently of the R : G ratio. These differential interactions of the receptor with different G proteins may account for different functions triggered by D_2 dopamine receptors.

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