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Agonist Regulation of D_2 Dopamine Receptor/G Protein Interaction

EVIDENCE FOR AGONIST SELECTION OF G PROTEIN SUBTYPE*

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The D_2 dopamine receptor has been expressed in Sf21 insect cells together with the G proteins G_o and G_{i2}, using the baculovirus system. Expression levels of receptor and G protein (α, β, and γ subunits) in the two preparations were similar as shown by binding of [^{3}H]spiperone and quantitative Western blot, respectively. For several agonists, binding data were fitted best by a two-binding site model in either preparation, showing interaction of expressed receptor and G protein. For some agonists, binding to the higher affinity site was of higher affinity in D_2/G_o than in the D_2/G_{i2} preparation. Some agonists exhibited binding data that were best fitted by a two-binding site model in D_2/G_o and a one-binding site model in D_2/G_{i2}. Therefore, receptor/G protein interaction seemed to be stronger in the D_2/G_o preparation. Agonist stimulation of [^{35}S]GTPγS (guanosine 5′-3-*O*-(thio)triphosphate) binding in the two preparations also gave evidence for higher affinity D_2/G_o interaction. In the D_2/G_o preparation, agonist stimulation of [^{35}S]GTPγS binding occurred at higher potency for several agonists, and a higher stimulation (relative to dopamine) was achieved in D_2/G_o compared with D_2/G_{i2}. Some agonists were able to stimulate [^{35}S]GTPγS binding in the D_2/G_o preparation but not in D_2/G_{i2}. The extent of D_2 receptor selectivity for G_o over G_{i2} is therefore dependent on the agonist used, and thus agonists may stabilize different conformations of the receptor with different abilities to couple to and activate G proteins.

There is considerable interest in understanding the action mechanisms of agonists at receptors (1–3). Agonists must bind to receptors, and this may be characterized in terms of an affinity of agonist binding. Agonists must also activate the receptor and associated signaling systems, and this property is often referred to as efficacy. Efficacy is exhibited in terms of the maximal effect induced by the agonist and also in the EC_{50} of the agonist in activating the signaling system, which is often lower than the concentration of agonist which achieves half-maximal occupancy of the receptor.

For G protein-coupled receptors, an influential model of agonist action is the ternary complex model and its recent extensions (4–6). In this model the receptor exists in an inactive ground state, which may isomerize to a partially activated state (R*)[1] that is able to couple more efficiently to the G protein to form the coupled active species (R*G). The formation of R*G may occur spontaneously, but in the presence of an agonist both R* and R*G are stabilized, and the ternary complex (AR*G) is formed. Guanine nucleotide exchange (GDP/GTP) occurs in both the binary complex (R*G) and the ternary complex (AR*G). The binary and ternary complexes dissociate releasing αGTP and $\beta\gamma$ subunits of the G protein which can alter effector activity. The agonist may also influence ternary complex breakdown (7, 8) so that there are several places at which agonism is determined.

There is, however, evidence that some receptors may interact with more than one G protein so that influences on different signaling pathways can occur. If a receptor can interact with more than one G protein this may influence the potency of agonist action and the pattern of agonist effects, *i.e.* the pharmacological profile of the response observed through the different G proteins. For the 5HT1A serotonin receptor, it was shown that the receptor interacts preferentially with $G_i/G_o/G_z$ subtypes of G protein (9) and that the nature of the G protein subtype influenced the agonist selectivity of the response (10). This question was addressed more explicitly for the α_2-adrenergic receptor (11). Expression of $G\alpha_o$, together with the endogenous G proteins of NIH 3T3 cells, altered the agonist selectivity of the receptor; the partial agonists, oxymetazoline and clonidine, exhibited increased efficacy. The possibility that the pharmacological profile of the response depends on the nature of the G protein has been termed "agonist trafficking" (12).

The D_2 dopamine receptor has been shown to interact with different G proteins to influence different signaling events (13, 14). In one study, interaction with G_o has been shown to lead to inhibition of calcium channels, whereas interaction with G_i subtypes has been shown to lead to inhibition of adenylyl cyclase (15). Also, the two splice variants of the D_2 receptor (D_{2short} and D_{2long}) have been reported to interact with different G proteins (13), although a clear definition of the selectivity pattern has not emerged as yet. Furthermore, the relative efficacies of quinpirole and (+)-3-PPP are reversed when tested on the D_2 receptor in the striatum and the pituitary gland (16), suggesting agonist trafficking, possibly via different G proteins.

To investigate these phenomena we have expressed the D_2

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[1] The abbreviations used are: R*, receptor in partially activated state; R*G, binary complex of G protein coupled to activated receptor; AR*G, ternary complex of agonist and R*G; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; CHO, Chinese hamster ovary; NMDG, *N*-methyl D-glucamine; NPA, *N*-propyl norapomorphine; K_l, low affinity agonist dissociation constant; K_h, high affinity agonist dissociation constant; 3-PPP, 3-(3-hydroxyphenyl)-*N*-propylpiperidine.

dopamine receptor together with the G proteins G_o and G_{i2} in insect cells, using the baculovirus system (17). This system provides a powerful tool for the reconstitution of receptor/G protein interactions. Insect cells do not contain endogenous dopamine receptors, and interaction between recombinant receptors and the endogenous G proteins of the cells is minimal.

EXPERIMENTAL PROCEDURES

Materials

[*phenyl*-4-^{3}H]Spiperone (25Ci/mmol) was from Amersham Pharmacia Biotech, and [^{35}S]GTPγS (1,250 Ci/mmol) was from PerkinElmer Life Sciences. Antibodies specific for different G protein subunits were from Chemicon and Santa Cruz as indicated. Other reagents were obtained as indicated or were of the highest purity available from commercial suppliers.

Methods

Cell Culture—Sf21 cells were grown either in monolayers or in suspension, using shaker flasks (25–100-ml cultures) agitated at 116 rpm. Cells were cultured at 26 °C in TC100 medium supplemented with 8% fetal calf serum and 0.1% Pluronic F-68 (Life Technologies, Inc.). CHO cells expressing the long form of the rat D_2 dopamine receptor (18, 19) were grown in RPMI medium containing 5% fetal calf serum, 2 mM L-glutamine, and 2 mM active Geneticin.

Construction and Isolation of Recombinant Baculovirus and Expression of the D_2 Dopamine Receptor and G Protein Subunits in Sf21 Cells—The baculovirus transfer vector, containing the cDNA for the FLAG-tagged D_{2long} dopamine receptor, was constructed from three DNA fragments (20). The first fragment consisted of the generic baculovirus transfer nonfusion vector, pVL1392 (PharMingen), digested with *Pst*I and *Bam*HI. The second fragment was generated by polymerase chain reaction and comprised at its 5′-end, a *Pst*I restriction site, to facilitate ligation to the vector, an ATG start codon, immediately followed by DNA encoding the FLAG epitope and the first 116 amino acids of the rat D_{2long} receptor sequence, and at its 3′-end, an *Alw*44I restriction site to allow ligation to the final cDNA fragment. The final fragment was a 1.0-kilobase cDNA fragment, coding for the remaining amino acids of the dopamine receptor, and was excised from an existing baculovirus transfer vector containing receptor cDNA (pVL1392D2), using *Alw*44I and *Bam*HI. The sequence of the DNA corresponding to the polymerase chain reaction fragment and the three ligation sites was confirmed by dideoxy DNA sequencing using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical). Transfer of the FLAG-D_{2long} cDNA into the *Autographa californica* nuclear polyhedrosis virus genome in the form of BaculoGold (PharMingen) was achieved by cotransfecting Sf21 cells with plasmid DNA and BaculoGold in the presence of Lipofectin (Life Technologies, Inc.). Recombinant baculovirus was purified by a single round of plaque purification (17) and stocks amplified (100-ml cultures, multiplicity of infection = 0.1). For expression, cells were infected at a cell density of 1×10^6 cells/ml with recombinant baculovirus at a multiplicity of infection of 10. Baculoviruses containing G protein sequences were constructed as described (21).

Preparation of Washed Cell Homogenates—All operations were carried out at 0–4 °C. Sf21 cells were harvested 48 h after infection by centrifugation at 3,000 × *g* for 10 min and resuspended at ~5×10^7 cells/ml in 20 mM HEPES, pH 7.4, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and protease inhibitors (Boehringer COMPLETE ™). Sf21 cells were homogenized with 50 strokes of a Dounce homogenizer and centrifuged at 3,000 × *g* for 10 min. The supernatant was collected and centrifuged at 48,000 × *g* for 60 min, and the pellet was resuspended in 20 mM HEPES, pH 7.4, 10 mM EDTA, 1 mM EGTA, and protease inhibitors (Boehringer COMPLETE ™). The resulting washed membrane homogenates were stored at −80 °C until used for Western blot analysis or ligand binding assays.

Membrane preparations from CHO cells expressing D_2 dopamine receptors were made as described by Castro and Strange (18, 19).

Protein Determination—Protein was determined using the Lowry method (22), with bovine serum albumin as the standard.

Ligand Binding Assays—Binding to washed membrane homogenates (15–50 μg of protein) was assayed in triplicate using [*phenyl*-4-^{3}H]spiperone (25Ci/mmol; 0.1–5 nM for saturation analyses and 1 nM for competition assays). Except where indicated, assays were performed in a final volume of 1 ml of assay buffer: 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 6 mM $MgCl_2$, pH 7.4. In agonist binding assays, 100 μM dithiothreitol was added as an antioxidant. For substituted benzamide antagonists, the standard assay buffer was supplemented with 100 mM NaCl or *N*-methyl D-glucamine (NMDG) as indicated. Binding was measured in the presence of 3 μM (−)-butaclamol and (+)-butaclamol to define total and nonspecific binding, respectively, over a period of 180 min at 25 °C. Bound and free radioligands were separated by rapid filtration through GF/B filters on a Brandel cell harvester with four washes of 4 ml of phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 5 mM Na_2HPO_4, pH 7.4). Bound radioactivity was determined by liquid scintillation counting. Ligand binding data were analyzed by nonlinear least squares regression using the computer program GraphPad Prism (GraphPad Software Inc.).

In some saturation assays a total assay volume of 10 ml was employed. The protein amount was the same as in the 1-ml assays so that the protein concentration was 10-fold lower. The concentrations of other substances were the same as in the 1-ml assays, but the time of incubation was 7 h.

[^{35}S]GTPγS Binding Assays—In agonist stimulation experiments, 50 μg of cell membranes were incubated in triplicate with 10 μM GDP and increasing concentrations of agonist in a final volume of 0.9 ml of buffer (20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4) for 30 min at 30 °C as described by Gardner *et al*. (23–25). 0.1 ml of [^{35}S]GTPγS (1,250 Ci/mmol) was added to a final concentration of 100 pM and the incubation continued for a further 20 min. Basal levels of [^{35}S]GTPγS binding were defined as that in the absence of agonist. Incubations were terminated by rapid filtration through Whatman GF/B glass fiber filters using a Brandel cell harvester with four washes of 4 ml of phosphate-buffered saline, and radioactivity determined as above. When different agonists were tested, a 1 mM dopamine control was always present in the assay to allow relative efficacy determinations to be made.

In saturation binding experiments, 40 μg of cell membranes was incubated in triplicate with 10 μM GDP, 100 pM [^{35}S]GTPγS, 100 pM-100 nM GTPγS in the absence or presence of 1 mM dopamine in a final volume of 1 ml of buffer for 2 h at 30 °C. Dopamine-stimulated [^{35}S]GTPγS binding was obtained by subtraction, and total dopamine-stimulated GTPγS binding was determined as dpm bound × ([total GTPγS]/[[^{35}S]GTPγS]).

Determination of G Protein Level Using Quantitative Western Blot—Before analysis, proteins (Sf21 membranes or pure G protein subunits) were denatured by the addition of 10 μl of electrophoresis loading buffer (100 mM Tris-Cl, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol) and heated at 90 °C for 5 min. Sf21 membrane proteins (20–40 μg) and G protein standards were separated by SDS-polyacrylamide gel electrophoresis on 12% acrylamide gels. Samples were then transferred to nitrocellulose membranes using the Bio-Rad semidry transfer system. Nitrocellulose membranes were incubated for 1 h with 5% dried milk (w/v) in buffer (137 mM NaCl, 3 mM KCl, 25 mM Tris-Cl, 0.1% Tween). Membranes were then incubated overnight at 4 °C with single primary antibodies (monoclonal antibody 3073 anti-α_o, 1 μg/ml (Chemicon); C-10 anti-α_{1-3}, 1 μg/ml (Santa Cruz, see Fig. 2); monoclonal antibody 3077 anti-α_{i2}, 1 μg/ml (Chemicon, see Fig. 3); C-16 anti-β_1, 0.4 μg/ml (Santa Cruz); A-16 anti-γ_2, 0.4 μg/ml (Santa Cruz)) in buffer containing 5% dried milk (w/v). Membranes were washed five times with buffer (10 min each) and then incubated with secondary antibody (anti-mouse (α_o, α_{i2})/rabbit (α_{i1-3}, β_1, γ_2) immunoglobin horseradish peroxidase conjugate (Sigma, 1:5,000)) for 1 h. Membranes were then washed three times (10 min each) with buffer before exposure to equal volumes of Enhanced Chemiluminescence (ECL) detection reagents 1 and 2 (Amersham Pharmacia Biotech). Membranes were then wrapped in Clingfilm and exposed to Hybond-ECL x-ray film for between 30 s and 2 min. Densitometry was performed using a GS710 calibrated imaging densitometer (Bio-Rad), and data were analyzed using GraphPad Prism. Determinations of levels of G protein subunits were always performed using ECL exposures that ensured a linear dependence of band density on protein amount.

In some experiments membranes were extracted with 1% cholate, 1 M NaCl (10 mg of membranes/ml of cholate/NaCl) for 1 h at 4 °C. The mixture was centrifuged at 4,500 × *g* for 5 min at 4 °C, and the supernatant and pellet were collected. These were then analyzed using Western blotting as above, the pellet having been dissolved in 1% cholate, 1% Nonidet P-40, and 1 M NaCl.

RESULTS

Expression of D_2 Dopamine Receptors in Sf21 Cells—D_2 dopamine receptors were expressed in Sf21 insect cells using the baculovirus expression system. The expressed receptors were characterized using ligand binding with [^{3}H]spiperone. Saturation analyses of [^{3}H]spiperone binding (1-ml assay volume) gave a K_d of 145 pM (pK_d 9.84 ± 0.03, mean ± S.E., n = 3) and

TABLE I

Binding of drugs to D_2 dopamine receptors expressed in Sf21 and CHO cells

Competition experiments *versus* [^{3}H]spiperone for various substances were performed as described under "Experimental Procedures," and K_i values (pK_i ± S.E., K_i from three or more experiments) were derived from the best fit curves to one-binding site models.

Ligand	Sf21			CHO		
	pK_i ± S.E.		Fold affinity change	pK_i ± S.E.		Fold affinity change
	NMDG	Na^+		NMDG	Na^+	
	nM			*nM*		
[^{3}H]Spiperone	9.46 ± 0.04 (0.34)[a]	9.72 ± 0.05 (0.19)	1.8 ↑	10.02 ± 0.08 (0.09)	10.08 ± 0.13 (0.08)	1.0
(+)-Butaclamol	8.38 ± 0.10 (4.2)	8.37 ± 0.14 (4.3)	1.0	ND[b]	ND	ND
Nemonapride	7.83 ± 0.15 (15.0)	9.57 ± 0.09 (0.27)	55 ↑	8.89 ± 0.19 (1.3)	10.05 ± 0.19 (0.09)	14 ↑
Clozapine	6.32 ± 0.03 (480)	6.63 ± 0.07 (240)	2 ↑	6.58 ± 0.13 (260)	6.20 ± 0.05 (630)	2.0 ↓
Clebopride	5.89 ± 0.07 (1,300)	8.11 ± 0.05 (7.7)	165 ↑	7.07 ± 0.09 (85)	8.40 ± 0.11 (4.0)	21 ↑
Raclopride	5.79 ± 0.09 (1,600)	7.44 ± 0.27 (36.0)	45 ↑	ND	ND	ND
(−)-Sulpiride	5.15 ± 0.20 (7,100)	6.48 ± 0.16 (330)	22 ↑	ND	ND	ND
Metoclopramide	4.81 ± 0.05 (15,000)	6.68 ± 0.14 (210)	73 ↑	5.26 ± 0.13 (5,500)	6.93 ± 0.04 (120)	47 ↑
Tiapride	<4.11 (>77,000)	6.01 ± 0.15 (1,000)	73 ↑	ND	ND	ND
Remoxipride	<4.0 (>100,000)	5.66 ± 0.22 (2,200)	>29 ↑	<5.1 (>7,900)	5.95 ± 0.14 (1,100)	>7 ↑
Dopamine	3.98 ± 0.13 (100,000)	4.51 ± 0.10 (31,000)	3 ↑	ND	ND	ND

[a] Values in parentheses are K_i, in nM.
[b] ND, not determined.

a B_{max} of ~2 pmol/mg. When these assays were repeated in a 10-ml format a similar K_d was observed (171 pM (pK_d 9.77 ± 0.21, mean ± S.E., $n = 3$)). The similarity of the K_d values from 1-ml and 10-ml assays demonstrates that radioligand depletion artifacts are absent from the assays (26).

A series of antagonists exhibited competition curves *versus* [^{3}H]spiperone which were best fitted by one-binding site models. The derived K_i values are given in Table I for experiments using buffer containing sodium ions and where the sodium had been replaced by NMDG to maintain ionic strength. The rank order of K_i values is similar to that observed for the D_2 receptor expressed in other systems, so the receptor is being expressed with fidelity in the present experiments. The substituted benzamide antagonists, *e.g.* sulpiride, are sensitive to the removal of sodium ions in these assays. Some data are also given for these drugs when binding to D_2 receptors expressed in CHO cells. In the presence of sodium ions K_i values are similar for the receptor expressed in the two cell backgrounds, whereas upon removal of sodium ions, binding of substituted benzamide drugs is of lower affinity for the receptors expressed in Sf21 cells.

Competition binding experiments were also performed with the agonists *N*-propyl norapomorphine (NPA) and dopamine and, except in one experiment with NPA, the data fitted best to a single-binding site model, and there was no significant effect of the addition of 100 μM GTP (see Table III). Formation of receptor-G protein complexes cannot, therefore, be detected in this way. Also, dopamine stimulation of [^{35}S]GTPγS binding could not be detected in membranes expressing the D_2 receptor without exogenous G proteins, whereas (see below) this activity was clearly present in membranes expressing exogenous G protein. The D_2 dopamine receptor does not, therefore, interact strongly with the endogenous G proteins of Sf21 cells. Similarly, when the formyl peptide receptor was expressed in Sf 9 insect cells no agonist stimulation of [^{35}S]GTPγS binding to the endogenous G proteins could be detected (27).

Coexpression of D_2 Dopamine Receptors and G Proteins in Sf21 Cells—In these experiments the D_2 dopamine receptor was expressed in Sf21 cells together with G protein α, β, and γ subunits. The G protein α subunits (α_o and α_{i2}) were used because the D_2 receptor has been reported to interact with these (13). The β_1 and γ_2 subunits were used for all of the studies here because these subunits support coupling between several receptors and G protein α subunits (27–31). In preliminary experiments, different multiplicity of infection values for the different baculoviruses containing the four proteins were tested to obtain similar receptor expression levels and a high [^{35}S]GTPγS binding response to dopamine. Based on these findings (data not shown), in the experiments described below, multiplicity of infection values were used as follows: for membranes expressing $G\alpha_{i2}$, receptor/α_{i2}/β_1/γ_2–2/2/1/1; for membranes expressing $G\alpha_o$, receptor/α_o/β_1/γ_2–3/1/1/1.

The levels of D_2 receptor were determined in the membranes using saturation analyses with [^{3}H]spiperone (Fig. 1 and Table II). Levels of D_2 dopamine receptor were similar in the two preparations, and there was no significant difference in the radioligand affinity. The affinity for [^{3}H]spiperone binding was unaffected by the addition of 100 μM GTP in both preparations and was not significantly different from that for the receptor expressed alone.

The levels of G protein α, β, and γ subunits were determined by quantitative Western blot, and the levels were not significantly different in the two preparations (Table II). Representative blots are shown in Fig. 2. Levels of α subunits were also determined after extraction of the membranes with 1% cholate. In each preparation 60–70% of the α subunit was found in the cholate extract, suggesting that the majority of the expressed subunits were fully active (Fig. 3). [^{35}S]GTPγS saturation binding assays in the presence of dopamine were also performed in the two preparations, and these showed B_{max} and K_d values for [^{35}S]GTPγS binding which were not significantly different (Table II and Fig. 1).

Agonist Binding to D_2 Dopamine Receptors Coexpressed with G Proteins—The binding of agonists to D_2 dopamine receptors

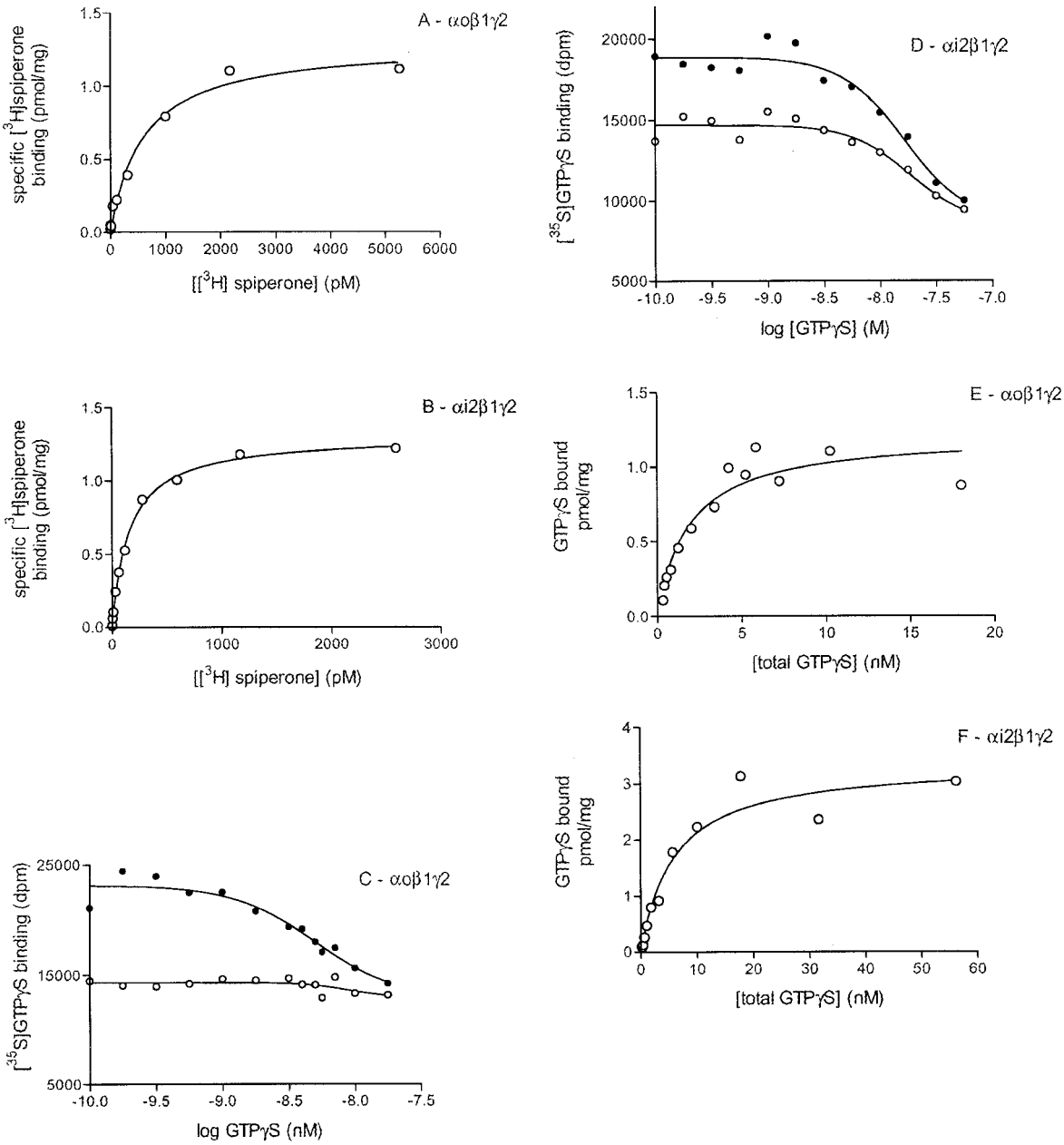

FIG. 1. **Saturation analyses for [^{3}H]spiperone and [^{35}S]GTPγS binding in membranes of Sf21 cells expressing D_2 dopamine receptors and G proteins.** [^{3}H]Spiperone and [^{35}S]GTPγS saturation binding experiments were performed on membranes expressing D_2 dopamine receptors and either G_o (*panels A*, *C*, and *E*) or G_{i2} (*panels B*, *D*, and *F*) as described under "Experimental Procedures." In *panels C* and *D* data are given for [^{35}S]GTPγS binding in the absence (○) and presence (●) of 1 mM dopamine. The dopamine-stimulated [^{35}S]GTPγS binding was determined by subtraction and was corrected for the added nonradioactive GTPγS as described under "Experimental Procedures" to give the data in *panels E* and *F*. Data are from representative experiments replicated as in Table I, and the curves in *panels A*, *B*, *E*, and *F* are best fit curves to one-site binding models.

was determined in competition with [^{3}H]spiperone in the preparations containing G_o and G_{i2}. Competition curves for several agonists (NPA, dopamine, (+)-3-PPP, *m*-tyramine), in both preparations, were best fitted by a two-site binding model with 20–30% higher affinity sites (Figs. 4 and 5; Table III). The proportion of higher affinity sites for a ligand did not differ significantly between the two preparations. Competition experiments for dopamine and NPA were also performed in the presence of 100 μM GTP, and competition curves under these conditions were best described by one-binding site models; the affinity in the presence of GTP was similar to that of the lower affinity site observed in the absence of GTP and also similar to that observed in preparations expressing receptor alone. For (−)-3-PPP, data obtained in the preparation containing G_o were also fitted best to a two-site model. For other agonists in both preparations (bromocriptine and *p*-tyramine) and for (−)-3-PPP in the preparation containing G_{i2} the competition curves were best fitted by a one-binding site model. When K_i values for the different sites were compared between the two preparations there were significant differences for some agonists (NPA, *m*-tyramine) at the higher affinity site, but for other agonists affinities at this site were not significantly different. Affinities at the lower affinity site and for the single affinity site seen for some agonists were not significantly different between the two preparations.

Stimulation of [^{35}S]GTPγS Binding by Agonists—G protein activation by agonists in the two preparations was assessed by determining agonist-stimulated [^{35}S]GTPγS binding (Fig. 6).

TABLE II

Expression of D_2 dopamine receptors and G proteins in Sf21 cells

D_2 dopamine receptors were expressed together with G protein subunits as described under "Experimental Procedures," and saturation binding analyses using [^{3}H]spiperone were performed to determine levels of D_2 receptor. Binding parameters (K_d and B_{max}) were derived from the data, and values are expressed as the mean ± S.E. (3). G protein levels were determined using quantitative Western blot and are expressed as the mean ± S.E. (3–4). Neither D_2 receptor nor G protein levels were significantly different in the two preparations ($p > 0.05$). [^{35}S]GTPγS saturation binding assays were performed, and K_d and B_{max} values are given; these were not significantly different between the two preparations ($p > 0.05$).

Preparation	[^{3}H]Spiperone binding			G protein Western blot			[^{35}S]GTPγS saturation binding	
	pK_d ± S.E. (K_d)		B_{max} control				pK_d ± S.E. (K_d)	B_{max}
	Control	+ GTP (100 μM)		α-subunit	β_1-subunit	γ_2-subunit		
			pmol/mg		*pmol/mg*			*pmol/mg*
$D_2/\alpha_o/\beta_1/\gamma_2$	10.01 ± 0.12 (98 pM)	9.74 ± 0.26 (180 pM)	1.16 ± 0.08	410 ± 140	110 ± 50	38 ± 13	8.80 ± 0.17 (1.6 nM)	1.34 ± 0.30
$D_2/\alpha_{i2}/\beta_1/\gamma_2$	9.78 ± 0.02 (160 pM)	9.67 ± 0.09 (220 pM)	1.11 ± 0.07	180 ± 60	130 ± 40	34 ± 15	8.34 ± 0.12 (4.6 nM)	2.61 ± 0.91

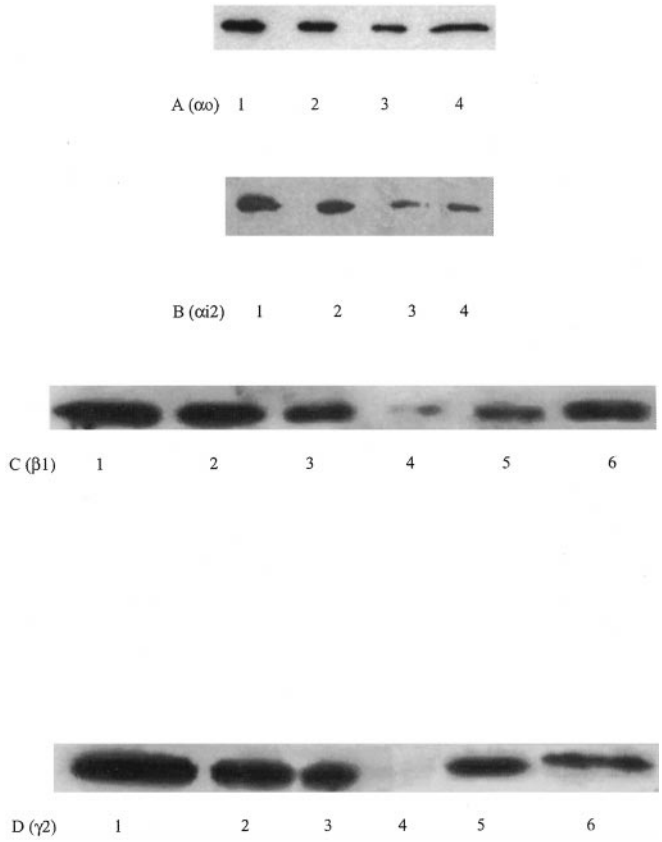

FIG. 2. **Determination of G protein levels by quantitative Western blot.** Samples of membranes expressing receptor and G proteins were analyzed by Western blot together with known amounts of pure G protein α, β, or γ subunit as described under "Experimental Procedures." Representative blots are shown for the preparations containing G_o and G_{i2}. The amount of G protein expressed was calculated, and the mean values from replicate experiments are given in Table II. In *panels A* and *B lanes 1–3* contain 0.5, 0.25, and 0.1 μg of pure G protein α subunit, respectively (*panel A*, α_o; *panel B*, α_{i2}), and *lane 4* contains 20 μg of membrane protein. In *panel C* (*lanes 1–4*) 0.3, 0.2, 0.1, and 0.05 μg of pure βγ dimer, respectively, was analyzed, and *lanes 5* and *6* contain 20 μg of membrane protein (*lane 5*, G_o; *lane 6*, G_{i2}), and the blot was probed for the β_1 subunit. In *panel D* (*lanes 1–4*) 0.15, 0.05, 0.025, and 0.0125 μg of pure βγ dimer, respectively, was analyzed, and *lanes 5* and *6* contain 40 μg of membrane protein (*lane 5*, G_o; *lane 6*, G_{i2}), and the blot was probed for the γ_2 subunit.

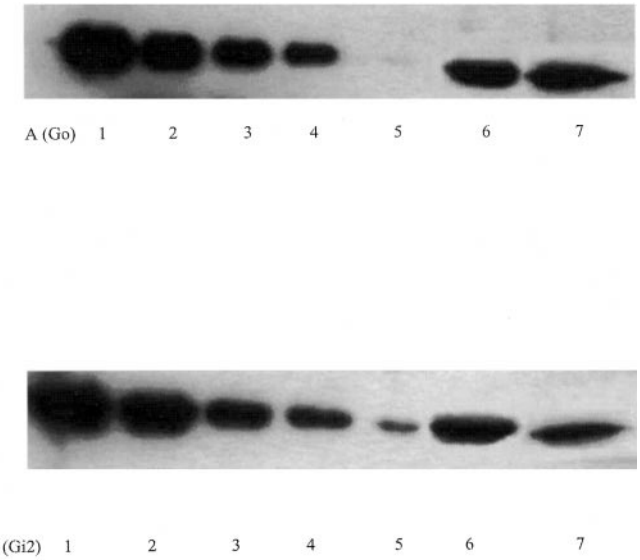

FIG. 3. **Extraction of G protein α subunits by cholate.** Membranes from preparations expressing G_o or G_{i2} were extracted with 1% cholate as described under "Experimental Procedures," and the levels of G protein α subunit were determined as in Fig. 2. In *panels A* and *B*, *lanes 1–5* contain 1, 0.5, 0.1, 0.05, and 0.01 μg of pure G protein α subunit, respectively (*panel A*, α_o; *panel B*, α_{i2}), and *lanes 6* and *7* contain, respectively, the supernatant and pellet from the cholate extract (equivalent to 100 μg of membrane protein). The distribution of α subunit in the two preparations was: G_o supernatant, 68 ± 5%; pellet, 32 ± 5%; G_{i2} supernatant, 59 ± 8%; pellet, 41 ± 8% (mean ± S.E. (3)).

These assays were conducted in the presence of 10 μM GDP to suppress basal [^{35}S]GTPγS binding and to observe agonist stimulation of [^{35}S]GTPγS binding over the basal level (23–25). Basal levels of [^{35}S]GTPγS binding may be high in this system because of the high levels of G protein α subunit expression. Under these conditions (*i.e.* in the presence of 10 μM GDP), full agonists lead to an approximate doubling of the rate of [^{35}S]GTPγS binding relative to the basal rate in both preparations. The EC_{50} values and maximal effects for a range of agonists are given in Table IV, and there are significant differences between the preparations containing G_o and G_{i2}. Several compounds stimulated [^{35}S]GTPγS binding to the same or greater extent than dopamine in both preparations. Four agonists (*m*-tyramine, *p*-tyramine, (+)-3-PPP, (−)-3-PPP), gave greater maximal stimulation in the preparation containing G_o compared with the preparation containing G_{i2}. Indeed, two of the compounds (*p*-tyramine and (−)-3-PPP) were unable to stimulate [^{35}S]GTPγS binding in the preparation containing G_{i2}. In addition to these differences in maximal stimulation, there were also significant differences (4–16-fold) in the EC_{50} values for the stimulation of [^{35}S]GTPγS binding between the two preparations for all the compounds tested, with the exception of bromocriptine.

DISCUSSION

In this study we have expressed the D_2 dopamine receptor together with the G proteins G_o and G_{i2} in insect cells using the

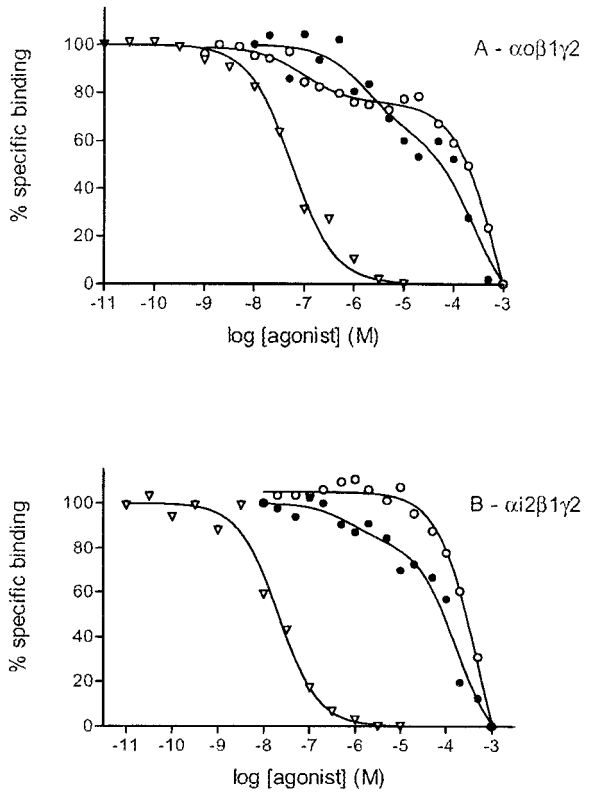

FIG. 4. **Binding of agonists to membranes of Sf21 cells expressing D_2 dopamine receptors and G proteins.** The binding of dopamine (●), bromocriptine (▽), and (−)-3-PPP (○) to membranes expressing D_2 receptor and either G_o (*panel A*) or G_{i2} (*panel B*) was determined in competition *versus* [^{3}H]spiperone as described under "Experimental Procedures." Data shown are from representative experiments replicated as in Table II, and the curves are the best fit curves to one-site (R/G_o, ▽; R/G_{i2}, ▽, ○) or two-site models (R/G_o, ○, ●; R/G_{i2}, ●).

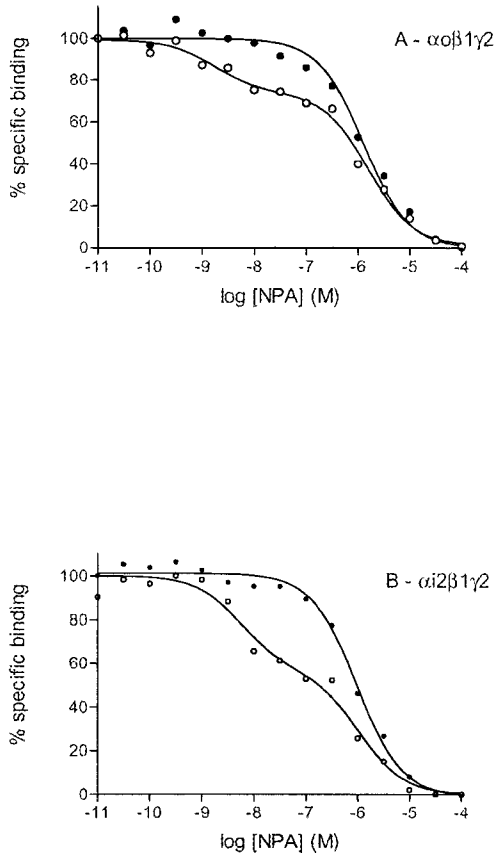

FIG. 5. **Binding of agonists to membranes of Sf21 cells expressing D_2 dopamine receptors and G proteins.** The binding of NPA to membranes expressing D_2 receptor and either G_o (*panel A*) or G_{i2} (*panel B*) was determined in competition *versus* [^{3}H]spiperone in the absence (○) or presence (●) of 100 μM GTP as described under "Experimental Procedures." Data shown are from representative experiments replicated as in Table II, and the curves are the best fit curves to one-site (●) or two-site models (○).

baculovirus system. We show that the D_2 receptor interacts more strongly with G_o than G_{i2} and that this influences the functional selectivity of agonist signaling. We also show that the extent of the selectivity of the interaction between the D_2 receptor and G_o or G_{i2} depends on the agonist used. Thus, agonists may stabilize different conformations of the receptor with different abilities to interact with and activate G proteins. This is the first study to address this issue for the D_2 dopamine receptor in a fully defined system.

The levels of receptor (R) and G protein (G) subunits (α_o/α_{i2}, and β_1 and γ_2) in the preparations expressing G_o and G_{i2} were determined using saturation analysis with the radioligand [^{3}H]spiperone and quantitative Western blot, respectively. The levels of α subunit detected were similar to those reported in other studies on expression of receptors and G_i or G_s proteins in insect cells (27, 32). The γ_2 subunit was expressed at lower levels than either the β_1 or α subunits and so may limit the levels of G protein heterotrimers. Membranes were also extracted with 1% cholate because this has been proposed to extract active G protein (33–36); in each preparation 60–70% of the α subunit was extractable. Based on these values and the limiting level of γ_2 subunit, there was a ratio of heterotrimeric G protein to receptor of ~20-fold in both preparations. This ratio is comparable with to obtained in other studies on expression of receptors and G proteins. G/R ratios of ~30–100 have been reported in insect cells (27, 31, 32), and G/R ratios of ~50 have been reported for α_2 adrenergic receptors in platelets (37) and ~100 for β-adrenergic receptors in lymphoma cells (38).

[^{35}S]GTPγS saturation binding assays were performed in the two preparations. These assays have been used by others to assess G protein levels (see, *e.g.* Ref. 39). In the present system, [^{35}S]GTPγS saturation binding assays gave similar values for the level of dopamine-stimulated [^{35}S]GTPγS binding in the two preparations. The levels determined by [^{35}S]GTPγS binding are low compared with the numbers of G proteins measured by quantitative Western blot. This is probably because there is a high concentration of GDP in the [^{35}S]GTPγS binding assays which reduces the binding of the radioligand substantially. At the highest concentrations of GTPγS used, there is more than a 100-fold excess of GDP, and therefore it is not surprising that the levels of α subunits detected by Western blotting are roughly 100-fold higher than detected in the [^{35}S]GTPγS binding assays. Nevertheless, in the present study, based on these different determinations, the levels of receptor and G protein subunits were similar in the membranes expressing G_o and G_{i2}. The two preparations are, therefore, comparable, and any differences between the preparations are unlikely to be caused either by receptor or G protein numbers.

Agonist binding in the membranes expressing receptor and G protein (G_o, G_{i2}) could, for many agonists, be resolved into contributions from sites of higher and lower affinity in similar proportions in the two preparations. This shows that the expressed D_2 receptor and G proteins are able to interact. For two of the agonists (dopamine, NPA), GTP abolished the higher affinity binding site. The affinity seen in the presence of GTP was similar to both the lower affinity site seen in the absence of GTP and the affinity for these agonists seen in a preparation containing receptor alone. These data follow the predicted be-

TABLE III

Agonist binding to D_2 dopamine receptors expressed in Sf21 cells

Competition experiments *versus* [^{3}H]spiperone were used to derive the binding parameters from the best fit curves (K_i from one-binding site models and K_h, K_l, and percent high affinity sites from two-binding site models) for experiments using membranes expressing D_2 receptors and G proteins. Data are expressed as the mean ± S.E. from three or more experiments. In preparations of membranes that had been infected only with the baculovirus coding for D_2 receptor, the following values were obtained: dopamine −GTP (4.80 ± 0.13 (16 μM)) +GTP (4.83 ± 0.08 (15 μM)); NPA −GTP (7.51 ± 0.19 (31 nM)) +GTP (7.11 ± 0.10 (77 nM)) (competition curves fit best to a one binding site model).

Ligand	Preparation					
	$D_2/\alpha_o/\beta_1/\gamma_2$			$D_2/\alpha_{i2}/\beta_1/\gamma_2$		
	pK_h (mean ± S.E.) (K_h)	pK_l (mean ± S.E.) (K_l)	% High affinity sites	pK_h (mean ± S.E.) (K_h)	pK_l (mean ± S.E.) (K_l)	% High affinity sites
	nM			*nM*		
Bromocriptine		9.09 ± 0.20 (0.82)			8.97 ± 0.03 (1.1)	
Dopamine	7.12 ± 0.20 (76)	4.68 ± 0.13 (21,000)	23 ± 8	7.15 ± 0.17 (70)	4.40 ± 0.24 (40,000)	19 ± 5
Dopamine + GTP (100 μM)		4.69 ± 0.14 (20,000)			4.93 ± 0.04 (12,000)	
NPA	10.24 ± 0.23 (0.06)	7.47 ± 0.37 (34)	33 ± 9	9.44 ± 0.08 (0.36)	7.22 ± 0.16 (61)	33 ± 6
NPA + GTP (100 μM)		7.40 ± 0.17 (40)			7.49 ± 0.32 (32)	
(+)-3-PPP	7.97 ± 0.29 (11)	4.55 ± 0.09 (28,000)	22 ± 6	7.74 ± 0.29 (18)	4.32 ± 0.10 (48,000)	19 ± 4
(−)-3-PPP	7.73 ± 0.10 (19)	4.96 ± 0.01 (11,000)	19 ± 5		4.80 ± 0.03 (16,000)	
m-Tyramine	7.60 ± 0.18 (25)	4.79 ± 0.30 (16,000)	36 ± 9	6.76 ± 0.36 (170)	>100,000	31 ± 4
p-Tyramine		4.27 ± 0.03 (54,000)			4.13 ± 0.04 (74,000)	

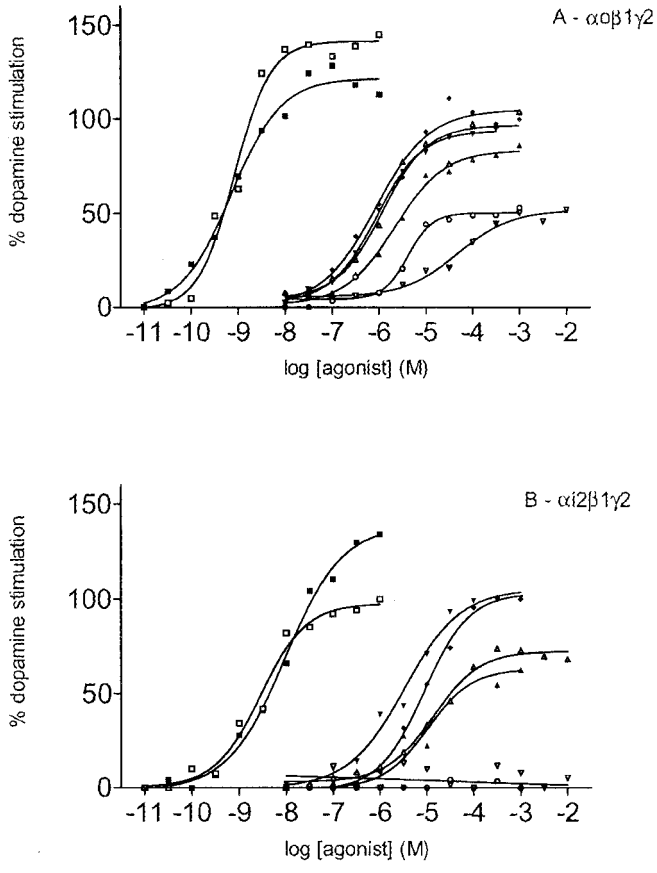

FIG. 6. **Stimulation of [^{35}S]GTPγS binding by agonists in membranes of Sf21 cells expressing D_2 dopamine receptors and G proteins.** The stimulation of [^{35}S]GTPγS binding by agonists was determined as described under "Experimental Procedures" in membranes expressing D_2 receptor and either G_o (*panel A*) or G_{i2} (*panel B*). Agonists used were as follows: bromocriptine (□), dopamine (♦), NPA (■), quinpirole (▼), *m*-tyramine (△), *p*-tyramine (▽), (−)-3-PPP (○), and (+)-3-PPP (▼). The data are representative stimulation curves replicated as in Table IV.

havior of a system that conforms to a ternary complex model with an excess of receptor over G protein (40, 41). The data on the levels of receptor and G protein in the membranes show, however, that there is an excess of G protein over receptor of about 20-fold. Similar discrepancies between inferred and measured R/G ratios have been noted in other systems. It has been proposed (37, 42) that receptors and G proteins may not interact freely and that there may be microdomains with different amounts of receptor and G protein. Alternatively, the ternary complex models are an oversimplification and receptor and G protein may form oligomers with properties different from the predictions of the models (43).

Two observations from the ligand binding studies suggest that there may be a greater affinity of the D_2 receptor for G_o than for G_{i2} when occupied by several agonists. First, the affinity of the higher affinity site is higher in the preparation containing G_o, for *m*-tyramine and NPA. This affinity difference should reflect the affinity of R/G coupling, given that the ground state affinity of the receptor is similar in the two preparations. Also, (−)-3-PPP is unable to stabilize the higher affinity state in the preparation containing G_{i2} but can do so in the preparation containing G_o. In agreement with these findings, differences in agonist affinity for one receptor coupled to different G proteins have been described by others (11, 44, 45).

A range of agonists was used to stimulate [^{35}S]GTPγS binding in the two preparations to assess G protein activation. Maximal agonist effects (relative to dopamine) were greater in the preparation containing G_o, and some agonists (*p*-tyramine, (−)-3-PPP) were unable to stimulate [^{35}S]GTPγS binding at all in the preparation containing G_{i2}. The potencies of agonists to stimulate [^{35}S]GTPγS binding were also generally greater in the preparation containing G_o, with the exception of bromocriptine. These data suggest that there is a more productive interaction between the D_2 receptor and G_o. The affinity of the interaction between receptor and G protein may contribute to this, as suggested above from the ligand binding data. The pattern of agonist binding and potencies in [^{35}S]GTPγS binding assays is very similar in the preparation containing G_o compared with that seen for the D_2 receptor expressed in CHO cells

TABLE IV

Agonist stimulation of [^{35}S]GTPγS binding via D_2 dopamine receptors expressed in Sf21 cells

The stimulation of [^{35}S]GTPγS binding in membranes expressing D_2 receptors and G proteins was determined as described under "Experimental Procedures." The maximum response (relative to dopamine) and the EC_{50} were determined. Data are expressed as the mean ± S.E. from three or more experiments.

Ligand	Preparation			
	$D_2/\alpha_o/\beta_1/\gamma_2$		$D_2/\alpha_{i2}/\beta_1/\gamma_2$	
	pEC_{50} ± S.E. (EC_{50})	% Maximal dopamine response	pEC_{50} ± S.E. (EC_{50})	% Maximal dopamine response
	nM		*nM*	
Bromocriptine	8.82 ± 0.17 (1.5)	145 ± 13	8.70 ± 0.09 (2.0)	97 ± 15
Dopamine	6.05 ± 0.20 (890)	100	4.84 ± 0.10 (14,000)	100
NPA	8.75 ± 0.22 (1.8)	113 ± 9	7.90 ± 0.08 (13)	134 ± 15
(+)-3-PPP	5.45 ± 0.12 (3,600)	86 ± 1	4.84 ± 0.14 (15,000)	64 ± 5
(−)-3-PPP	5.83 ± 0.27 (1,500)	53 ± 5	ND[a]	0
Quinpirole	6.29 ± 0.15 (510)	95 ± 11	5.57 ± 0.15 (2,700)	101 ± 6
m-Tyramine	5.73 ± 0.15 (1,900)	104 ± 13	4.80 ± 0.05 (16,000)	68 ± 6
p-Tyramine	4.40 ± 0.23 (40,000)	52 ± 5	ND	0

[a] ND, not determined.

TABLE V

Agonist signaling parameters

Parameters were derived from data in Tables III and IV. K_h and K_l are respectively the dissociation constants for the higher and lower affinity agonist binding sites as in Table III. EC_{50} is the concentration of agonist which gives a 50% maximal response in the [^{35}S]GTPγS binding assays as in Table IV.

Ligand	Preparation			
	K_l/K_h		K_l/EC_{50}	
	$D_2/\alpha_o/\beta_1/\gamma_2$	$D_2/\alpha_{i2}/\beta_1/\gamma_2$	$D_2/\alpha_o/\beta_1/\gamma_2$	$D_2/\alpha_{i2}/\beta_1/\gamma_2$
Bromocriptine	1	1	0.6	0.6
Dopamine	270	570	23	2.8
NPA	600	170	19	4.8
(+)-3-PPP	2,600	2,700	7.9	3.3
(−)-3-PPP	600	1	7.5	
m-Tyramine	660	>600	8.7	6.4
p-Tyramine	1	1	1.3	

(23–25). The present system is, therefore, behaving similarly to a system in which the receptor couples exclusively with endogenous mammalian G proteins.

To understand the differences between the two preparations in more detail, the data were analyzed to provide the K_l/EC_{50} ratio (ratio of agonist binding dissociation constant to agonist potency) (Table V). The K_l/EC_{50} ratio (or amplification ratio (24, 25, 46)) indicates the extent to which agonist activation of a response occurs at lower concentrations than agonist binding to the receptor and so is a measure of receptor/G protein activation. The K_l/EC_{50} ratio of the agonists is greater in the preparation containing G_o than in the preparation containing G_{i2}, providing a further indication that there is a more productive interaction between the D_2 receptor and G_o.

For the preparation containing G_o, greater K_l/EC_{50} ratios are generally observed for the agonists that give greater maximal effects for stimulation of [^{35}S]GTPγS binding. In this preparation, therefore, the two measures of efficacy, agonist maximal effect and K_l/EC_{50}, are in agreement for a range of compounds. For the preparation containing G_{i2}, lower values of K_l/EC_{50} are seen for several agonists, but for two of the agonists, *p*-tyramine and (−)-3-PPP, no agonism is seen at all. For these compounds, binding to the receptor appears to be insufficient to stabilize receptor/G protein interaction. In this preparation, therefore, receptor/G protein interaction is less efficient, and for some agonists there is a complete failure to signal. The data outlined earlier show that the affinity of the D_2 dopamine receptor is greater for G_o than for G_{i2}. This cannot be the only factor influencing the activation of the G proteins because otherwise a general reduction in signaling efficiency would be seen for all agonists tested when the lower affinity interaction is present, *i.e.* in the G_{i2} preparation. This suggests that different agonists are able to stabilize different conformations of the receptor with different affinities for the G protein and different functional activities in the ternary complex rather than there being differential stabilization of the same activated state by different agonists. As a result, the selectivity of the D_2 receptor for G_o over G_{i2} is dependent on the agonist used. The two agonists that show the greatest selectivity, *p*-tyramine and (−)-3-PPP, are both monohydroxylated compounds. It is interesting that for the α_{2A}-adrenergic receptor, catechol agonists, *e.g.* noradrenaline, lead to stimulation of both G_i- and G_s-dependent pathways, whereas monohydroxylated agonists, *e.g.* octopamine, lead only to activation of G_i-dependent pathways (47).

Further evidence that agonists may regulate the activity of the ternary complex comes from analysis of the K_l/K_h ratio (ratio of low affinity and high affinity agonist dissociation constants). The K_l/K_h ratio was derived from the ligand binding data (Table V) because this has been proposed to be an index of the ability of the agonist to stabilize receptor/G protein coupling (see, *e.g.* Ref. 40). There is no clear relationship between

the maximal effects of the agonists in [^{35}S]GTPγS binding assays and the K_l/K_h ratio. Therefore, stabilization of receptor/G protein coupling is not a clear predictor of agonist efficacy, and similar results were seen in other studies on the D_2 receptor expressed in CHO cells (24, 25). Agonists may, therefore, influence the activity of the ternary complex as well as its formation (7, 8, 24, 25).

The behavior of bromocriptine provides further support for the idea that agonists stabilize different receptor conformations. Bromocriptine is a full agonist on both preparations, and its potency (EC_{50}) and binding affinity (K_l) are similar in each preparation, leading to identical K_l/EC_{50} ratios, in contrast to the other agonists tested. This suggests that the bromocriptine-receptor complex has a similar affinity for the two G proteins. Bromocriptine is an unusual compound in that its binding to D_2 receptors conforms to a single-site binding model (Table II) and is insensitive to guanine nucleotides (24, 25, 48). It has been suggested that this is because bromocriptine is able to stabilize a conformation of the receptor which is close to the conformation in the active receptor-G protein complex (49, 50) so that there is little energy gain in coupling to the G protein. This would be consistent with the present findings in that the bromocriptine-receptor complex does not show any discrimination between G_o and G_{i2}, and the G protein is fully active in each case. The close agreement between K_l and EC_{50} supports this contention.

In conclusion, we have shown that the D_2 dopamine receptor has a greater affinity for the G protein G_o than for G_{i2}. Activation of G_o occurs with higher potencies for agonists and greater relative efficacies for partial agonists, and this is in agreement with the findings of Yang and Lanier (11) for the α_2-adrenergic receptor. The data do not provide evidence for agonist trafficking in that there are no clear reversals of agonist potency. The pattern of agonist potencies is, however, different for the two receptor-G protein combinations. Therefore, the extent of selectivity of the D_2 dopamine receptor for the two G proteins (G_o, G_{i2}) depends on the agonist used. Different agonists, therefore, stabilize different conformations of the receptor which can couple to and activate G proteins differentially.

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